PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: COMPOSITE ANTIBODIES OF HUMAN SUBGROUP IV LIGHT CHAIN CAPABLE OF BINDING TO TAG-72

(57) Abstract

This invention concerns a subset of composite Hum4 V_L, $V_H\alpha$TAG antibody with high affinities to a high molecular weight, tumor-associated sialylated glycoprotein antigen (TAG-72) of human origin. These antibodies have variable regions with (1) V_L segments derived from the human subgroup IV germline gene and (2) a V_H segment which is capable of combining with the V_L to form a three dimensional structure having the ability to bind TAG-72. *in vivo* methods of treatment and diagnostic assay using these composite antibodies is also disclosed.

Best Available Copy

COMPOSITE ANTIBODIES OF HUMAN SUBGROUP IV LIGHT CHAIN
CAPABLE OF BINDING TO TAG-72

The present invention is directed to the fields
of immunology and genetic engineering.

The following information is provided for the
purpose of making known information believed by the
applicants to be of possible relevance to the present
invention. No admission is necessarily intended, nor
should be construed, that any of the following
information constitutes prior art against the present
invention.

Antibodies are specific immunoglobulin (Ig)
polypeptides produced by the vertebrate immune system in
response to challenges by foreign proteins,
glycoproteins, cells, or other antigenic foreign
substances. The binding specificity of such
polypeptides to a particular antigen is highly refined.
with each antibody being almost exclusively directed to
the particular antigen which elicited it.

Two major methods of generating vertebrate
antibodies are presently utilized:generation *in situ* by
the mammalian B lymphocytes and generation in cell
culture by B-cell hybrids. Antibodies are generated *in*

situ as a result of the differentiation of immature B
lymphocytes into plasma cells (see Gough (1981), <u>Trends
in Biochem Sci</u>, 6:203 (1981). Even when only a single
antigen is introduced into the immune system for a
particular mammal, a uniform population of antibodies
does not result, i.e., the response is polyclonal.

The limited but inherent heterogeneity of
polyclonal antibodies is overcome by the use of
hybridoma technology to create "monoclonal" antibodies
in cell cultures by B cell hybridomas (see Kohler and
Milstein (1975), <u>Nature</u>, 256:495-497). In this process,
a mammal is injected with an antigen, and its relatively
short-lived, or mortal, splenocytes or lymphocytes are
fused with an immortal tumor cell line. The fusion
produces hybrid cells or "hybridomas" which are both
immortal and capable of producing the genetically-coded
antibody of the B cell.

In many applications, the use of monoclonal
antibodies produced in non-human animals is severely
restricted where the monoclonal antibodies are to be
used in humans. Repeated injections in humans of a
"foreign" antibody, such as a mouse antibody, may lead
to harmful hypersensitivity reactions, i.e., an anti-
idiotypic, or human anti-mouse antibody (HAMA)
response, (see Shawler *et al.* (1985), <u>Journal of
Immunology</u>, 135:1530-1535. and Sear *et al.*, <u>J. Biol. Resp.
Modifiers</u>, 3:138-150).

Various attempts have already been made to
manufacture human-derived monoclonal antibodies by using
human hybridomas (see Olsson *et al.*, <u>Proc. Natl. Acad.
Sci. U.S.A.</u>, 77:5429 (1980) and Roder *et al.* (1986),
<u>Methods in Enzymology</u>, 121:140-167. Unfortunately,

yields of monoclonal antibodies from human hybridoma
cell lines are relatively low compared to mouse
hybridomas. In addition, human cell lines expressing
immunoglobulins are relatively unstable compared to
mouse cell lines, and the antibody producing capability
of these human cell lines is transient. Thus, while
human immunoglobulins are highly desirable, human
hybridoma techniques have not yet reached the stage
where human monoclonal antibodies with required
antigenic specificities can be easily obtained.

Thus, antibodies of nonhuman origin have been
genetically engineered. or "humanized". Humanized
antibodies reduce the HAMA response compared to that
expected after injection of a human patient with a mouse
antibody. Humanization of antibodies derived from
nonhumans, for example. has taken two principal forms,
i.e., chimerization where non-human regions of
immunoglobulin constant sequences are replaced by
corresponding human ones (see for example. USP 4,816,567
to Cabilly *etal.*, Genentech) and grafting of
complementarity determining regions (CDR) into human
framework regions (FR) (see European Patent Office
Application (EPO) 0 239 400 to Winter). Some
researchers have produced Fv antibodies (USP 4,642,334
to Moore, DNAX) and single chain Fv (SCFV) antibodies
(see USP 4,946,778 to Ladner, Genex).

The above patent applications only show the
production of antibody fragments in which some portion
of the variable domains is coded for by nonhuman 7 gene
regions. Humanized antibodies to date still retain
various portions of light and heavy chain variable
regions of nonhuman origin: the chimeric. Fv and single
chain Fv antibodies retain the entire variable region of

nonhuman origin and CDR-grafted antibodies retain CDR of nonhuman origin.

Such nonhuman-derived regions are expected to elicit an immunogenic reaction when administered into a human patient (see Brüggemann *et al.* (1989), *J. Exp. Med.*, 170:2153-2157; and Lo Buglio (1991), Sixth International Conference on Monoclonal Antibody Immunoconjugates for Cancer, San Diego, Ca). Thus, it is most desirable to obtain a human variable region which is capable of binding to a selected antigen.

One known human carcinoma tumor antigen is tumor-associated glycoprotein-72 (TAG-72), as defined by monoclonal antibody B72.3 (see Thor *et al.* (1986) *Cancer Res.*, 46:3118-3124; and Johnson, *et al.* (1986), *Cancer Res.*, 46:850-857). TAG-72 is associated with the surface of certain tumor cells of human origin, specifically the LS174T tumor cell line (American Type Culture Collection (ATCC) No. CL 188), which is a variant of the LS180 (ATCC No. CL 187) colon adeno-carcinoma line.

Numerous murine monoclonal antibodies have been developed which have binding specificity for TAG-72. Exemplary murine monoclonal antibodies include the "CC" (colon cancer) monoclonal antibodies, which are a library of murine monoclonal antibodies developed using TAG-72 purified on an immunoaffinity column with an immobilized anti-TAG-72 antibody, B72.3 (ATCC HB-8108) (see EP 394277, to Schlom *et al.*, National Cancer Institute). Certain CC antibodies were deposited with the ATCC: CC49 (ATCC No. HB 9459); CC83 (ATCC No. HB 9453); CC46 (ATCC No. HB 9458); CC92 (ATCC No. HB 9454); CC30 (ATCC NO. HB 9457); CC11 (ATCC No. 9455) and CC15

(ATCC No. HB 9460). Various antibodies of the CC series have been chimerized (see, for example, EPO 0 365 997 to Mezes *et al.*, The Dow Chemical Company).

It is thus of great interest to develop
5 antibodies against TAG-72 containing a light and/or heavy chain variable region(s) derived from human antibodies. However, the prior art simply does not teach recombinant and immunologic techniques capable of routinely producing an anti-TAG-72 antibody in which the
10 light chain and/or the heavy chain variable regions have specificity and affinity for TAG-72 and which are derived from human sequences so as to elicit expectedly low or no HAMA response. It is known that the function of an immunoglobulin molecule is dependent on its three
15 dimensional structure, which in turn is dependent on its primary amino acid sequence. A change of a few or even one amino acid can drastically affect the binding function of the antibody can drastically affect its the
20 bidning affinity of the antibody, i.e., the resultant antibodies are generally presumed to be a non-specific immunoglobulin (NSI), i.e., lacking in antibody character, (see, for example, USP 4,816,567 to Cabilly *et al.*, Genentech).
25

Surprisingly, the present invention is capable of meeting many of these above mentioned needs and provides a method for supplying the desired antibodies. For example, in one aspect, the present invention
30 provides a cell capable of expressing a composite antibody having binding specificity for TAG-72, said cell being transformed with (a) a DNA sequence encoding at least a portion of a light chain variable region (V_L) effectively homologous to the human Subgroup IV germline gene (Hum4 V_L); and a DNA sequence segment encoding at

-6-

least a portion of a heavy chain variable region (V_H) capable of combining with the V_L into a three dimensional structure having the ability to bind to TAG-72.

5   In another aspect, the present invention provides a composite antibody or antibody having binding specificity for TAG-72, comprising (a) a DNA sequence encoding at least a portion of a light chain (V_L) variable region effectively homologous to the human

10   Subgroup IV germline gene (Hum4 V_L); and a DNA sequence segment encoding at least a portion of a heavy chain variable region (V_H) capable of combining with the V_L into a three dimensional structure having the ability to

15   bind TAG-7.

The invention further includes the aforementioned antibody alone or conjugated to an imaging marker or therapeutic agent. The invention also

20   includes a composition comprising the aforementioned antibody in unconjugated or conjugated form in a pharmaceutically acceptable, non-toxic, sterile carrier.

The invention is also directed to a method for

25   *in vivo* diagnosis of cancer which comprises administering to an animal containing a tumor expressing TAG-72 a pharmaceutically effective amount of the aforementioned composition for the *in situ* detection of carcinoma lesions.

30
The invention is also directed to a method for intraoperative therapy which comprises (a) administering to patient containing a tumor expressing TAG-72 a pharmaceutically effective amount of the aforementioned

composition, whereby the tumor is localized, and (b) excising the localized tumors.

Additionally, the invention also concerns a process for preparing and expressing a composite antibody. Some of these processes are as follows. A process which comprises transforming a cell with a DNA sequence encoding at least a portion of a light chain variable region (V_L) effectively homologous to the human Subgroup IV germline gene (Hum4 V_L); and a DNA sequence segment encoding at least a portion of a heavy chain variable region (V_H) which is capable of combining with the V_L to form a three dimensional structure having the ability to bind to TAG-72. A process for preparing a composite antibody or antibody which comprises culturing a cell containing a DNA sequence encoding at least a portion of a light chain variable region (V_L) effectively homologous to the human Subgroup IV germline gene (Hum4 V_L); and a DNA sequence segment encoding at least a portion of a heavy chain variable region (V_H) capable of combining with the V_L into a three dimensional structure having the ability to bind to TAG-72 under sufficient conditions for the cell to express the immunoglobulin light chain and immuno-globulin heavy chain. A process for preparing an antibody conjugate comprising contacting the aforementioned antibody or antibody with an imaging marker or therapeutic agent.

Description of the Drawings

Figure 1 illustrates a basic immunoglobulin structure.

Figure 2 illustrates the nucleotide sequences of $V_H\alpha TAG$, CC46 V_H, CC49 V_H, CC83 V_H and CC92 V_H.

Figure 3 illustrates the amino acid sequences of $V_H\alpha TAG$, CC46 V_H, CC49 V_H, CC83 V_H and CC92 V_H.

Figure 4 illustrates the V_H nucleotide and amino acid sequences of antibody B17X2.

Figure 5 illustrates the mouse germline J-H genes from pNP9.

Figure 6 illustrates the plasmid map of p49 g1-2.3.

Figure 7 illustrates the plasmid map of p83 g1-2.3.

Figure 8 illustrates the entire sequence of HUMVL(+) and HUMVL(-).

Figure 9 illustrates the human J4 (HJ4) nucleotide sequence and amino acid sequence.

Figure 10 illustrates the nucleotide sequences, and the amino acid sequences of Hum4 V_L, *ClaI-Hind*III segment.

Figure 11 illustrates a schematic representation of the human germline Subgroup IV V_L gene (Hum4 V_L), as the target for the PCR.

Figure 12 shows the results of agarose gel electrophoresis of the PCR reaction to obtain the Hum4 V_L gene.

Figure 13 illustrates the restriction enzyme map of pRL1000, and precursor plasmids pSV2neo,

pSV2neo-101 and pSV2neo-102. "X" indicates where the *Hind*III site of pSV2neo has been destroyed.

Figure 14 illustrates a polylinker segment made by synthesizing two oligonucleotides: CH(+) and CH(-).

Figure 15 illustrates a primer, NEO102SEQ, used for sequencing plasmid DNA from several clones of pSV2neo-102.

Figure 16 illustrates an autoradiogram depicting the DNA sequence of the polylinker region in pSV2neo-102.

Figure 17 illustrates a partial nucleotide sequence segment of pRL1000.

Figure 18 illustrates the restriction enzyme map of pRL1001.

Figure 19 illustrates an autoradiogram of DNA sequence for pRL1001 clones.

Figure 20 illustrates a competition assay for binding to TAG-using a composite Hum4 V_L, $V_H\alpha$TAG antibody.

Figure 21 illustrates a general DNA construction of a single chain, composite Hum4 V_L, $V_H\alpha$TAG.

Figure 22 illustrates the nucleotide sequence and amino acid sequence of SCFV1.

Figure 23 shows the construction of plasmid pCGS515/SCFV1.

Figure 24 shows the construction of plasmid pSCFV31.

Figure 25 shows the construction of E. coli SCFV expression plasmids containing Hum4 V_L.

Figure 26 shows the DNA sequence and amino acid sequence of Hum4 V_L-CC49V_H SCFV present in pSCFVUHH.

Figure 27 shows the construction plasmid pSCFV UHH and a schematic of a combinatorial library of V_H genes with Hum4 V_L.

Figure 28 illustrates the nucleotide sequence of FLAG peptide adapter in pATDFLAG.

Figure 29 illustrates the construction of pATDFLAG, pHumVL-HumVH (X) and pSC49FLAG.

Figure 30 illustrates the nucleotide and amino acid sequences of pSC49FLAG.

Detailed Description of the Invention

Nucleic acids, amino acids, peptides, protective groups, active groups and so on, when abbreviated, are abbreviated according to the IUPAC IUB (Commission on Biological Nomenclature) or the practice in the fields concerned.

The basic immunoglobulin structural unit is set forth in Figure 1. The terms "constant" and "variable" are used functionally. The variable regions of both light (V_L) and heavy (V_H) chains determine binding recognition and specificity to the antigen. The constant region domains of light (C_L) and heavy (C_H) chains confer important biological properties such as

antibody chain association, secretion, transplacental
mobility, complement binding, binding to Fc receptors
and the like.

5 The immunoglobulins of this invention have been
developed to address the problems of the prior art. The
methods of this invention produce, and the invention is
directed to, composite antibodies. By "composite
antibodies" is meant immunoglobulins comprising variable
regions not hitherto found associated with each other in
10 nature. By, "composite Hum4 V_L, V_H antibody" means an
antibody or immunoreactive fragment thereof which is
characterized by having at least a portion of the V_L
region encoded by DNA derived from the Hum4 V_L germline
gene and at least a portion of a V_H region capable of
15 combining with the V_L to form a three dimensional
structure having the ability to bind to TAG-72.

The composite Hum4 V_L, V_H antibodies of the
20 present invention assume a conformation having an
antigen binding site which binds specifically and with
sufficient strength to TAG-72 to form a complex capable
of being isolated by using standard assay techniques
(e.g., enzyme-linked immunosorbent assay (ELISA),
25 radioimmunoassay (RIA), or flourescence-activated cell
sorter analysis (FACS), immunohistochemistry and the
like). Preferably, the composite Hum4 V_L, V_H antibodies
of the present invention have an antigen binding
affinity or avidity greater than 10^5 M^{-1}, more
30 preferably greater than 10^6 M^{-1} and most preferably
greater than 10^8 M^{-1}. For a discussion of the
techniques for generating and reviewing immunoglobulin
binding affinities see Munson (1983), Methods Enzymol.,

92:543-577 and Scatchard (1949), <u>Ann. N.Y. Acad. Sci.</u>, 51:660-672.

Human antibody kappa chains have been classified into four subgroups on the basis of invariant amino acid sequences (see, for example, Kabat *et al.* (1991), <u>Sequences of Proteins of Immunological Interest</u> (4th ed.), published by The U.S. Department of Health and Human Services). There appear to be approximately 80 human V_K genes, but only one Subgroup IV V_K gene has been identified in the human genome (see Klobeck, *et al.* (1985), <u>Nucleic Acids Research</u>, 13:6516-6528). The nucleotide sequence of Hum4 V_L is set forth in Kabat *et al.* (1991), *supra*; and Wang *et al.* (1973), <u>Nature</u>, 243:126-127.

It has been found, quite surprisingly, that an immunoglobulin having a light chain with at least a portion of the V_L encoded by a gene derived from Hum4 V_L may, if combined with a suitable V_H, have binding specificity for TAG-72.

The type of J_L gene segment selected is not critical to the invention, in that it is expected that any J_L, if present, can associate with the Hum4 V_L. The present invention obviously contemplates the Hum4 V_L in association with a human J_K sequence. The five human J_K sequences are set forth in Heiter *et al.* (1982), <u>The Journal of Biological Chemistry</u>, 357:1516-1522. However, the present invention is not intended to be limited to the human J_K. The present invention specifically contemplates the Hum4 V_L in association with any of the at least six human J_λ genes (see Hollis *et al.* (1982), <u>Nature</u>, 296:321-325).

An exemplary technique for engineering the Hum4 V_L with selected J_L segments includes synthesizing a primer having a so-called "wagging tail", that does not hybridize with the target DNA; thereafter, the sequences are amplified and spliced together by overlap extension

5   (see Horton *et al.* (1989), <u>Gene</u>, 77:61-68).

The C_L of the composite Hum4 V_L, V_H antibodies is not critical to the invention. To date, the Hum4 V_L has only been reported as having been naturally

10   rearranged with the single C_k gene (see Heiter *et al.* (1980), Cell, 22:197-207). However, the present invention is not intended to be limited to the C_k light chain constant domain. That is, the C_L gene segment may also be any of the at least six C_λ genes (see Hollis *et*

15   *al.*, *supra*).

The DNA encoding the heavy chain variable region consists roughly of a heavy chain variable (V_H) gene sequence, a heavy chain diversity (D_H) gene

20   sequence, and a heavy chain joining (J_H) gene sequence.

The present invention is directed to any V_H capable of combining with a light chain variable region effectively homologous to the light chain variable

25   region encoded by the human Subgroup IV germline gene, to form a three dimensional structure having the ability to bind to TAG-72.

The choice of heavy chain diversity (D_H)

30   segment and the heavy chain joining (J_H) segment of the composite Hum4 V_L, V_H antibody are not critical to the present invention. Obviously, human and murine D_H and J_H gene segments are contemplated, provided that a given combination does not significantly decrease binding to

WO 93/12231

-14-

TAG-72. Specifically, when utilizing CC46 V_H, CC49 V_H, CC83 V_H and CC92 V_H, the composite Hum4 V_L, VH antibody will be designed to utilize the D_H and J_H segments which naturally associated with those V_H of the respective hybridomas (see Figures 2 and 3). Exemplary murine and

5 human D_H and J_H sequences are set forth in Kabat *et al.* (1991), *supra*. An exemplary technique for engineering such selected D_H and J_H segments with a V_H sequence of choice includes synthesizing selected oligonucleotides, annealing and ligating in a cloning procedure (see,

10 Horton *et al.*, *supra*).

In a specific embodiment the composite Hum4 V_L, V_H antibody will be a "composite Hum4 V_L, $V_H\alpha$TAG antibody", means an antibody or immunoreactive fragment

15 thereof which is characterized by having at least a portion of the V_L region encoded by DNA derived from the Hum4 V_L germline gene and at least a portion of the V_H region encoded by DNA derived from the $V_H\alpha$TAG germline

20 gene, which is known in the art (see, for example, EPO 0 365 997 to Mezes *et al.*, the Dow Chemical Company). Figure 2 shows the nucleotide sequence of $V_H\alpha$TAG, and the nucleotide sequences encoding the V_H of the CC46, CC49, CC83 and CC92 antibodies, respectively. Figure 3

25 shows the corresponding amino acid sequences of $V_H\alpha$TAG, CC46 V_H, CC49 V_H, CC83 V_H and CC92 V_H.

A comparison of the nucleotide and amino acid sequences of $V_H\alpha$TAG, CC46 V_H, CC49 V_H, CC83 V_H and CC92

30 V_H shows that those CC antibodies are derived from $V_H\alpha$TAG. Somatic mutations occurring during productive rearrangement of the V_H derived from $V_H\alpha$TAG in a B cell gave rise to some nucleotide changes that may or may not

result in a homologous amino acid change between the productively rearranged hybridomas (see, EPO 0 365 997).

Because the nucleotide sequences of $V_H\alpha TAG$ and Hum4 V_L germline genes have been provided herein, the present invention is intended to include other antibody genes which are productively rearranged from the $V_H\alpha TAG$ germline gene. Other antibodies encoded by DNA derived from $V_H\alpha TAG$ may be identified by using a hybridization probe made from the DNA or RNA of the $V_H\alpha TAG$ or rearranged genes containing the recombined $V_H\alpha TAG$. Specifically, the probe will include of all or a part of the $V_H\alpha TAG$ germline gene and its flanking regions. By "flanking regions" is meant to include those DNA sequences from the 5' end of the $V_H\alpha TAG$ to the 3' end of the upstream gene, and from 3' end of the $V_H\alpha TAG$ to the 5' end of the downstream gene.

The CDR from the variable region of antibodies derived from $V_H\alpha TAG$ may be grafted onto the FR of selected V_H, i.e., FR of a human antibody (see EPO 0 239 400 to Winter). For example, the cell line, B17X2, expresses an antibody utilizing a variable light chain encoded by a gene derived from Hum4 V_L and a variable heavy chain which makes a stable V_L and V_H combination (see Marsh *et al.* (1985), <u>Nucleic Acids Research</u>, 13:6531-6544; and Polke *et al.* (1982), <u>Immunobiol.</u> 163:95-109. The nucleotide sequence of the V_H chain for B17X2 is shown in Figure 4. The B17X2 cell line is publicly available from Dr. Christine Polke, Universitäts-Kinderklinik, Josef-Schneider-Str. 2, 8700 Würzburg, FRG). B17X2 is directed to N-Acetyl-D-Glucosamine and is not specific for TAG-72.

However, consensus sequences of antibody
derived from the CDR1 of V$_H$αTAG (amino acid residues 31
to 35 of Figure 3) may be inserted into B17X2 (amino
acid residues 31 to 37 of Figure 4) and the CDR2 of
V$_H$αTAG (amino residues 50 to 65 of Figure 3) may be
5   inserted into B17X2 (amino acid residues 52 to 67 of
Figure 4). The CDR3 may be replaced by any D$_H$ and J$_H$
sequence which does not affect the binding of the
antibody for TAG-72 but, specifically, may be replaced
by the CDR3 of an antibody having its V$_H$ derived from
10   V$_H$αTAG, e.g., CC46, CC49, CC83 and CC92. Exemplary
techniques for such replacement are set forth in Horton
et al., supra.

15   The C$_H$ domains of immunoglobulin heavy chain
derived from V$_H$αTAG genes, for example may be changed to
a human sequence by known techniques (see, USP 4,816,567
to Cabilly, Genentech). C$_H$ domains may be of various
complete or shortened human isotypes, i.e., IgG (e.g.,
20   IgG$_1$, IgG$_2$, IgG$_3$, and IgG$_4$), IgA (e.g., IgA1 and IgA2),
IgD, IgE, IgM, as well as the various allotypes of the
individual groups (see Kabat *et al.* (1991), *supra*).

Given the teachings of the present invention,
25   it should be apparent to the skilled artisan that human
V$_H$ genes can be tested for their ability to produce an
anti-TAG-72 immunoglobilin combination with the Hum4 V$_L$
gene. The V$_L$ may be used to isolate a gene encoding for
a V$_H$ having the ability to bind to TAG-72 to test myriad
30   combinations of Hum4 V$_L$ and V$_H$ that may not naturally
occur in nature, e.g., by generating a combinatorial
library using the Hum4 V$_L$ gene to select a suitable V$_H$.
Examples of these enabling technologies include
screening of combinatorial libraries of V$_L$-V$_H$
combinations using an Fab or single chain antibody

(SCFV) format expressed on the surfaces of fd phage (Clackson, *et al.* (1991), <u>Nature</u>, 352:624-628), or using a λ phage system for expression of Fv's or Fabs (Huse, *et al.* (1989), <u>Science</u>, 246:1275-1281). However, according to the teachings set forth herein, it is now possible to clone SCFV antibodies in *E. coli*, and express the SCFVs as secreted soluble proteins. SCFV proteins produced in *E. coli* that contain a Hum4 V_L gene can be screened for binding to TAG-72 using, for example, a two-membrane filter screening system (Skerra, *et al.* (1991), <u>Analvtical Biochemistry</u>, 196:151-155).

The desired gene repertoire can be isolated from human genetic material obtained from any suitable source, e.g., peripheral blood lymphocytes, spleen cells and lymph nodes of a patient with tumor expressing TAG-72. In some cases, it is desirable to bias the repertoire for a preselected activity, such as by using as a source of nucleic acid, cells (source cells) from vertebrates in any one of various stages of age, health and immune response.

Cells coding for the desired sequence may be isolated, and genomic DNA fragmented by one or more restriction enzymes. Tissue (e.g., primary and secondary lymph organs, neoplastic tissue, white blood cells from peripheral blood and hybridomas) from an animal exposed to TAG-72 may be probed for selected antibody producing B cells. Variability among B cells derived from a common germline gene may result from somatic mutations occurring during productive rearrangement.

Generally, a probe made from the genomic DNA of a germline gene or rearranged gene can be used by those

-18-

skilled in the art to find homologous sequences from
unknown cells. For example, sequence information
obtained from Hum4 V_L and $V_H\alpha$TAG may be used to generate
hybridization probes for naturally-occurring rearranged
V regions, including the 5' and 3' nontranslated
5   flanking regions. The genomic DNA may include
naturally-occurring introns for portions thereof,
provided that functional splice donor and splice
acceptor regions had been present in the case of
10   mammalian cell sources.

Additionally, the DNA may also be obtained from
a cDNA library. mRNA coding for heavy or light chain
variable domain may be isolated from a suitable source,
15 . either mature B cells or a hybridoma culture, employing
standard techniques of RNA isolation. The DNA or amino
acids also may be synthetically synthesized and
constructed by standard techniques of annealing and
ligating fragments (see Jones, *et al.* (1986), <u>Nature</u>,
20   321:522-525; Reichmann *et al.*, (1988), Nature, 332:323-
327; Sambrook *et al.* (1989), *supra* and Merrifield *et al.*
(1963), <u>J. Amer. Chem. Soc.</u>, 85:2149-2154). Heavy and
light chains may be combined *in vitro* to gain antibody
activity (see Edelman, *et al.* (1963), <u>Proc. Natl. Acad.</u>
25 <u>Sci. USA</u>, 50:753).

The present invention also contemplates a gene
library of $V_H\alpha$TAG homologs, preferably human homologs of
$V_H\alpha$TAG. By "homolog" is meant a gene coding for a V_H
30   region (not necessarily derived from, or even
effectively homologous to, the $V_H\alpha$TAG germline gene)
capable of combining with a light chain variable region
effectively homologous to the light chain variable
region encoded by the human Subgroup IV germline gene.

to form a three dimensional structure having the ability to bind to TAG-72.

Preferably, the gene library is produced by a primer extension reaction or combination of primer extension reactions as described herein. The V$_H$αTAG homologs are preferably in an isolated form, that is, substantially free of materials such as, for example, primer extension reaction agents and/or substrates, genomic DNA segments, and the like. The present invention thus is directed to cloning the V$_H$αTAG-coding DNA homologs from a repertoire comprised of polynucleotide coding strands, such as genomic material containing the gene expressing the variable region or the messenger RNA (mRNA) which represents a transcript of the variable region. Nucleic acids coding for V$_H$αTAG-coding homologs can be derived from cells producing IgA, IgD, IgE, IgG or IgM, most preferably from IgM and IgG, producing cells.

The V$_H$αTAG-coding DNA homologs may be produced by primer extension. The term "primer" as used herein refers to a polynucleotide whether purified from a nucleic acid restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complimentary to a nucleic acid strand is induced, i.e., in the presence of nucleotides and an agent for polymerization such as DNA polymerase, reverse transcriptase and the like, and at a suitable temperature and pH.

Preferably, the V$_H$αTAG-coding DNA homologs may be produced by polymerase chain reaction (PCR) amplification of double stranded genomic or cDNA, wherein two

primers are used for each coding strand of nucleic acid
to be exponentially amplified. The first primer becomes
part of the nonsense (minus or complementary) strand and
hybridizes to a nucleotide sequence conserved among V_H
(plus) strands within the repertoire. PCR is described
5   in Mullis et al. (1987), Meth. Enz., 155:335-350; and PCR
Technology, Erlich (ed.) (1989). PCR amplification of
the mRNA from antibody-producing cells is set forth in
Orlandi et al. (1989), Proc. Natl. Acad. Sci.. USA,
10   86:3387-3837.

According to a preferred method, the $V_H\alpha$TAG-
coding DNA homologs are connected via linker to form a
SCFV having a three dimensional structure capable of
15   binding TAG-72. The SCFV construct can be in a V_L-L-V_H
or V_H-L-V_L configuration. For a discussion of SCFV see
Bird et al. (1988), Science, 242:423-426. The design of
suitable peptide linker regions is described in U.S.
Patent No. 4,704,692 to Ladner et al.. Genex.
20

The nucleotide sequence of a primer is selected
to hybridize with a plurality of immunoglobulin heavy
chain genes at a site substantially adjacent to the
$V_H\alpha$TAG-coding DNA homolog so that a nucleotide sequence
25   coding for a functional (capable of binding) polypeptide
is obtained. The choice of a primer's nucleotide
sequence depends on factors such as the distance on the
nucleic acid from the region coding for the desired
receptor, its hybridization site on the nucleic acid
30   relative to any second primer to be used, the number of
genes in the repertoire it is to hybridize to, and the
like. To hybridize to a plurality of different nucleic
acid strands of $V_H\alpha$TAG-coding DNA homolog, the primer

must be a substantial complement of a nucleotide sequence conserved among the different strands.

The peptide linker may be coded for by the nucleic acid sequences that are part of the poly-nucleotide primers used to prepare the various gene libraries. The nucleic acid sequence coding for the peptide linker can be made up of nucleic acids attached to one of the primers or the nucleic acid sequence coding for the peptide linker may be derived from nucleic acid sequences that are attached to several polynucleotide primers used to create the gene libraries. Additionally, noncomplementary bases or longer sequences can be interspersed into the primer, provided the primer sequence has sufficient complemen-tarily with the sequence of the strand to be synthesized or amplified to non-randomly hybridize therewith and thereby form an extension product under polynucleotide synthesizing conditions (see Horton *et al.* (1989), Gene, 77:61-68).

Exemplary human V_H sequences from which complementary primers may be synthesized are set forth in Kabat *et al.* (1991), *supra*: Humphries *et al.* (1988), Nature, 331:446-449; Schroeder *et al.* (1990), Proc. Natl. Acad. Sci. USA, 87:6146-6150; Berman *et al.* (1988), EMBO Journal, 7:727-738; Lee *et al.* (1987), J. Mol. Biol., 195:761-768); Marks *et al.* (1991), Eur. J. Immunol., 21:985-991; Willems, *et al.* (1991), J. Immunol., 146:3646-3651; and Person *et al.* (1991), Proc Natl. Acad. Sci. USA, 88:2432-2436. To produce V_H coding DNA homologs, first primers are therefore chosen to hybridize to (i.e. be complementary to) conserved regions within the J region, CH1 region, hinge region, CH2 region, or CH3 region of immunoglobulin genes and the like. Second primers are

therefore chosen to hydribidize with a conserved
nucleotide sequence at the 5' end of the V_HaTAG-coding
DNA homolog such as in that area coding for the leader
or first framework region.

5   Alternatively, the nucleic acid sequences
coding for the peptide linker may be designed as part of
a suitable vector. As used herein, the term "expression
vector" refers to a nucleic acid molecule capable of
directing the expression of genes to which they are
10   operatively linked. The choice of vector to which a
V_HaTAG-coding DNA homologs is operatively linked depends
directly, as is well known in the art, on the functional
properties desired, e.g., replication or protein
expression, and the host cell (either procaryotic or
15   eucaryotic) to be transformed, these being limitations
inherent in the art of constructing recombinant DNA
molecules. In preferred embodiments, the eucaryotic
cell expression vectors used include a selection marker
that is effective in an eucaryotic cell, preferably a
20   drug resistant selection marker.

        Expression vectors compatible with procaryotic
cells are well known in the art and are available from
25   several commercial sources. Typical of vector plasmids
suitable for procaryotic cells are pUC8, pUC9, pBR322,
and pBR329 available from BioRad Laboratories,
(Richmond, CA), and pPL and pKK223 available from
Pharmacia, (Piscataway, NJ).
30

        Expression vectors compatible with eucaryotic
cells, preferably those compatible with vertebrate
cells, can also be used. Eucaryotic cell expression
vectors are well known in the art and are available from
several commercial sources. Typically, such vectors are

provided containing convenient restriction sites for insertion of the desired DNA homologue. Typical of vector plasmids suitable for eucaryotic cells are pSV2neo and pSV2gpt (ATCC), pSVL and pKSV-10 (Pharmacia), pBPV-1/PML2d (International Biotechnologies, Inc.), and pTDT1 (ATCC).

The use of viral expression vectors to express the genes of the $V_H\alpha$TAG-coding DNA homologs is also contemplated. As used herein, the term "viral expression vector" refers to a DNA molecule that includes a promoter sequences derived from the long terminal repeat (LTR) region of a viral genome. Exemplary phage include λ phage and fd phage (see, Sambrook, *et al.* (1989), Molecular Cloning: A Laboratory Manual. (2nd ed.), and McCafferty *et al.* (1990), Nature, 6301:552-554.

The population of $V_H\alpha$TAG-coding DNA homologs and vectors are then cleaved with an endonuclease at shared restriction sites. A variety of methods have been developed to operatively link DNA to vectors via complementary cohesive termini. For instance, complementary cohesive termini can be engineered into the $V_H\alpha$TAG-coding DNA homologs during the primer extension reaction by use of an appropriately designed polynucleotide synthesis primer, as previously discussed. The complementary cohesive termini of the vector and the DNA homolog are then operatively linked (ligated) to produce a unitary double stranded DNA molecule.

The restriction fragments of Hum4 V_L-coding DNA and the $V_H\alpha$TAG-coding DNA homologs population are randomly ligated to the cleaved vector. A diverse,

random population is produced with each vector having a V_HαTAG-coding DNA homolog and Hum4 V_L-coding DNA located in the same reading frame and under the control of the vector's promoter.

5   The resulting single chain construct is then introduced into an appropriate host to provide amplification and/or expression of a composite Hum4 V_L, V_HαTAG homolog single chain antibody. Transformation of appropriate cell hosts with a recombinant DNA molecule

10   of the present invention is accomplished by methods that typically depend on the type of vector used. With regard to transformation of procaryotic host cells, see, for example. Cohen *et al.* (1972), Proceedings National

15   Academy of Science. USA. 69:2110; and Sambrook, *et al.* (1989), *supra.* With regard to the transformation of vertebrate cells with retroviral vectors containing rDNAs, see for example. Sorge *et al.* (1984), Mol. Cell. Biol., 4:1730-1737; Graham *et al.* (1973), Virol., 52:456;

20   and Wigler *et al.* (1979), Proceedings National Academy of Sciences. USA, 76:1373-1376.

       Exemplary prokaryotic strains that may be used as hosts include *E.coli, Bacilli,* and other entero-

25   bacteriaceae such as *Salmonella typhimurium,* and various *Pseudomonas.* Common eukaryotic microbes include *S. cerevisiae* and *Pichia pastoris.* Common higher eukaryotic host cells include Sp2/0, VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 and

30   MDCK cell lines. Furthermore. it is now also evident that any cell line producing Hum4 V_L, e.g., the B17X2 human cell line. can be used as a recipient human cell line for introduction of a V_H gene complementary to the Hum4 V_L which allows binding to TAG-72. For example, the B17X2 heavy chain may be genetically modified to not

produce the endogenous heavy chain by well known methods; in this way, glycosylation patterns of the antibody produced would be human and not non-human derived.

5   Successfully transformed cells, i.e., cells containing a gene encoding a composite Hum4 V_L, V$_H\alpha$TAG homolog single chain antibody operatively linked to a vector, can be identified by any suitable well known technique for detecting the binding of a receptor to a
10   ligand. Preferred screening assays are those where the binding of the composite Hum4 V_L, V$_H\alpha$TAG homolog single chain antibody to TAG-72 produces a detectable signal, either directly or indirectly. Screening for productive
15   Hum4 V_L and V$_H\alpha$TAG homolog combinations, or in other words, testing for effective antigen binding sites to TAG-72 is possible by using for example, a radiolabeled or biotinylated screening agent, e.g., antigens, anti- bodies (e.g., B72.3, CC49, CC83, CC46, CC92, CC30, CC11
20   and CC15) or anti-idiotypic antibodies (see Huse *et al.*, *supra*, and Sambrook *et al.*, *supra*); or the use of marker peptides to the NH$_2$- or COOH-terminus of the SCFV construct (see Hopp *et al.* (1988). Biotechnology, 6:1204- 1210).
25

      Of course, the Hum4 V_L-coding DNA and the V$_H\alpha$TAG-coding DNA homologs may be expressed as individual polypeptide chains (e.g.. Fv) or with whole or fragmented constant regions (e.g., Fab, and F(ab')$_2$).
30   Accordingly. the Hum4 V_L-coding DNA and the V$_H\alpha$TAG- coding DNA homologs may be individually inserted into a vector containing a C_L or C_H or fragment thereof, respectively. For a teaching of how to prepare suitable

vectors see EPO 0 365 997 to Mezes *et al.*, The Dow
Chemical Company.

DNA sequences encoding the light chain and
heavy chain of the composite Hum4 V_L, V_H antibody may be
inserted into separate expression vehicles, or into the
same expression vehicle. When coexpressed within the
same organism, either on the same or the different
vectors, a functionally active Fv is produced. When the
$V_H\alpha$TAG-coding DNA homolog and Hum4 V_L polypeptides are
expressed in different organisms, the respective
polypeptides are isolated and then combined in an
appropriate medium to form a Fv. See Greene *et al.*,
Methods in Molecular Biology, Vol. 9, Wickner *et al.*
(ed.); and Sambrook *et al.*, *supra*).

Subsequent recombinations can be effected
through cleavage and removal of the Hum4 V_L-coding DNA
sequence to use the $V_H\alpha$TAG-coding DNA homologs to
produce Hum4 V_L-coding DNA homologs. To produce a Hum4
V_L-coding DNA homolog, first primers are chosen to
hybridize with (i.e. be complementary to) a conserved
region within the J region or constant region of
immunoglobulin light chain genes and the like. Second
primers become part of the coding (plus) strand and
hybridize to a nucleotide sequence conserved among minus
strands. Hum4 V_L-coding DNA homologs are ligated into
the vector containing the $V_H\alpha$TAG-coding DNA homolog,
thereby creating a second population of expression
vectors. The present invention thus is directed to
cloning the Hum4 V_L-coding DNA homologs from a
repertoire comprised of polynucleotide coding strands,
such as genomic material containing the gene expressing
the variable region or the messenger RNA (mRNA) which
represents a transcript of the variable region. It is

thus possible to use an iterative process to define yet further, composite antibodies, using later generation $V_H\alpha TAG$-coding DNA homologs and Hum4 V_L-coding DNA homologs.

5   The present invention further contemplates genetically modifying the antibody variable and constant regions to include effectively homologous variable region and constant region amino acid sequences. Generally, changes in the variable region will be made
10   in order to improve or otherwise modify antigen binding properties of the receptor. Changes in the constant region of the antigen receptor will, in general, be made in order to improve or otherwise modify biological
15   properties, such as complement fixation, interaction with membranes, and other effector functions.

          "Effectively homologous" refers to the concept that differences in the primary structure of the
20   variable region may not alter the binding characteristics of the antigen receptor. Normally, a DNA sequence is effectively homologous to a second DNA sequence if at least 70 percent, preferably at least 80 percent, and most preferably at least 90 percent of the
25   active portions of the DNA sequence are homologous. Such changes are permissable in effectively homologous amino acid sequences so long as the resultant antigen receptor retains its desired property.

30   If there is only a conservative difference between homologous positions of sequences, they may be regarded as equivalents under certain circumstances. General categories of potentially equivalent amino acids are set forth below. wherein, amino acids within a group may be substituted for other amino acids in that group:

(1) glutamic acid and aspartic acid; (2) hydrophobic amino acids such as alanine, valine, leucine and isoleucine; (3) asparagine and glutamine; (4) lysine, arginine; and (5) threonine and serine.

5   Exemplary techniques for nucleotide replacement include the addition, deletion, or substitution of various nucleotides, deletion or substitution of various nucleotides, provided that the proper reading frame is maintained. Exemplary techniques include using

10 polynucleotide-mediated, site-directed mutagenesis, i.e., using a single strand as a template for extension of the oligonucleotide to produce a strand containing the mutation (see Zoller *et al.* (1982), <u>Nuc. Acids Res.</u>,

15 10:6487-6500; Norris et al. (1983), <u>Nuc. Acids Res.</u>, 11:5103-5112; Zoller et al. (1984), <u>DNA</u>, 3:479-488; Kramer *et al.* (1982), <u>Nuc. Acids Res.</u>, 10:6475-6485 and polymerase chain reaction, i.e., exponentially amplifying DNA *in vitro* using sequence specified oligo-

20 nucleotides to incorporate selected changes (see <u>PCR</u> <u>Technology: Principles and Applications for DNA</u> <u>Amplification</u>, Erlich, (ed.) (1989); and Horton *et al.* *supra*).

25   Further, the antibodies may have their constant region domain modified, ie., the C_L, CH_1, hinge, CH_2, CH_3 and/or CH_4 domains of an antibody polypeptide chain may be deleted, inserted or changed (see EPO 327 378 A1 to Morrison *et al.*, the Trustees of Columbia University;

30 USP 4,642,334 to Moore *et al.*, DNAX: and USP 4,704,692 to Ladner *et al.*, Genex).

       Once a final DNA construct is obtained, the composite Hum4 V_L, V_H antibodies may be produced in large quantities by injecting the host cell into the

peritoneal cavity of pristane-primed mice, and after an appropriate time (about 1-2 weeks), harvesting ascites fluid from the mice, which yields a very high titer of homogeneous composite Hum4 V_L, V_H antibodies, and

5 isolating the composite Hum4 V_L, V_H antibodies by methods well known in the art (see Stramignoni. *et al.* (1983), <u>Intl. J. Cancer</u>, 31:543-552). The host cell are grown *in vivo*, as tumors in animals, the serum or ascites fluid of which can provide up to about 50 mg/mL of

10 composite Hum4 V_L, V_H antibodies. Usually, injection (preferably intraperitoneal) of about 10^6 to 10^7 histocompatible host cells into mice or rats will result in tumor formation after a few weeks. It is possible to obtain the composite Hum4 V_L, V_H antibodies from a

15 fermentation culture broth of procaryotic and eucaryotic cells, or from inclusion bodies of *E.coli* cells (see Buckholz and Gleeson (1991), BIO/TECHNOLOGY, 9:1067-1072. The composite Hum4 V_L, V_H antibodies can then be collected and processed by well-known methods (see

20 generally, <u>Immunological Methods</u>, vols. I & II, eds. Lefkovits, I. and Pernis, B., (1979 & 1981) Academic Press, New York, N.Y.: and <u>Handbook of Experimental Immunology</u>, ed. Weir, D., (1978) Blackwell Scientific

25 Publications, St. Louis, MO.)

The composite Hum4 V_L, V_H antibodies can then be stored in various buffer solutions such as phosphate buffered saline (PBS), which gives a generally stable

30 antibody solution for further use.

<u>Uses</u>

The composite Hum4 V_L, V_H antibodies provide unique benefits for use in a variety of cancer treatments. In addition to the ability to bind

specifically to malignant cells and to localize tumors and not bind to normal cells such as fibroblasts, endothelial cells, or epithelial cells in the major organs, the composite Hum4 V_L, V_H antibodies may be used to greatly minimize or eliminate ANHA responses thereto.

5 Moreover, TAG-72 contains a variety of epitopes and thus it may be desirable to administer several different composite Hum4 V_L, V_H antibodies which utilize a variety of V_H in combination with Hum4 V_L.

10 Specifically, the composite Hum4 V_L, V_H antibodies are useful for, but not limited to, *in vivo* and *in vitro* uses in diagnostics, therapy, imaging and biosensors.

15 The composite Hum4 V_L, V_H antibodies may be incorporated into a pharmaceutically acceptable, non--toxic, sterile carrier. Injectable compositions of the present invention may be either in suspension or

20 solution form. In solution form the complex (or when desired the separate components) is dissolved in a pharmaceutically acceptable carrier. Such carriers comprise a suitable solvent, preservatives such as benzyl alcohol, if needed, and buffers. Useful solvents

25 include, for example, water. aqueous alcohols, glycols, and phosphonate or carbonate esters. Such aqueous solutions generally contain no more than 50 percent of the organic solvent by volume.

30 Injectable suspensions require a liquid suspending medium. with or without adjuvants, as a carrier. The suspending medium can be, for example, aqueous polyvinyl-pyrrolidone, inert oils such as vegetable oils or highly refined mineral oils, or aqueous carboxymethlycellulose. Suitable physio-

logically-acceptable adjuvants, if necessary to keep the complex in suspension, may be chosen from among thickeners such as carboxymethylcellulose, polyvinyl-pyrrolidone, gelatin, and the alginates. Many surfactants are also useful as suspending agents, for example, lecithin, alkylphenol, polyethylene oxide adducts, naphthalenesulfonates, alkylbenzenesulfonates, and the polyoxyethylene sorbitan esters. Many substances which effect the hydrophibicity, density, and surface tension of the liquid suspension medium can assist in making injectable suspensions in individual cases. For example, silicone antifoams, sorbitol, and sugars are all useful suspending agents.

Methods of preparing and administering conjugates of the composite Hum4 V_L, V_H antibody, and a therapeutic agent are well known to or readily determined. Moreover, suitable dosages will depend on the age and weight of the patient and the therapeutic agent employed and are well known or readily determined.

Conjugates of a composite Hum4 V_L, V_H antibody and an imaging marker may be administered in a pharma-ceutically effective amount for the *in vivo* diagnostic assays of human carcinomas, or metastases thereof, in a patient having a tumor that expresses TAG-72 and then detecting the presence of the imaging marker by appropriate detection means.

Administration and detection of the conjugates of the composite Hum4 V_L, V_H antibody and an imaging marker, as well as methods of conjugating the composite Hum4 V_L, V_H antibody to the imaging marker are accomplished by methods readily known or readily determined. The dosage of such conjugate will vary

depending upon the age and weight of the patient. Generally, the dosage should be effective to visualize or detect tumor sites, distinct from normal tissues. Preferably, a one-time dosage will be between 0.1 mg to 200 mg of the conjugate of the composite Hum4 V_L antibody and imaging marker per patient.

Examples of imaging markers which can be conjugated to the composite Hum4 V_L antibody are well known and include substances which can be detected by diagnostic imaging using a gamma scanner or hand held gamma probe, and substances which can be detected by nuclear magnetic resonance imaging using a nuclear magnetic resonance spectrometer.

Suitable, but not limiting, examples of substances which can be detected using a gamma scanner include ^{125}I, ^{131}I, ^{123}I, ^{111}In, ^{105}Rh, ^{153}Sm, ^{67}Cu, ^{67}Ga, ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re and ^{99m}Tc. An example of a substance which can be detected using a nuclear magnetic resonance spectrometer is gadolinium.

Conjugates of a composite Hum4 V_L, V_H antibodies and a therapeutic agent may be administered in a pharmaceutically effective amount for the *in vivo* treatment of human carcinomas, or metastases thereof, in a patient having a tumor that expresses TAG-72. A "pharmaceutically effective amount" of the composite Hum4 V_L antibody means the amount of said antibody (whether unconjugated. i.e., a naked antibody, or conjugated to a therapeutic agent) in the pharmaceutical composition should be sufficient to achieve effective binding to TAG-72.

Exemplary naked antibody therapy includes, for example, administering heterobifunctional composite Hum4 V_L, V_H antibodies coupled or combined with another antibody so that the complex binds both to the carcinoma and effector cells, e.g., killer cells such as T cells, or monocytes. In this method, the composite Hum4 V_L antibody-therapeutic agent conjugate can be delivered to the carcinoma site thereby directly exposing the carcinoma tissue to the therapeutic agent. Alternatively, naked antibody therapy is possible in which antibody dependent cellular cytoxicity or complement dependent cytotoxicity is mediated by the composite Hum4 V_L antibody.

Examples of the antibody-therapeutic agent conjugates which can be used in therapy include antibodies coupled to radionuclides, such as ^{131}I, ^{90}Y, ^{105}Rh, ^{47}Sc, ^{67}Cu, ^{212}Bi, ^{211}At, ^{67}Ga, ^{125}I, ^{186}Re, ^{188}Re, ^{177}Lu, ^{99m}Tc, ^{153}Sm, ^{123}I and ^{111}In; to drugs, such as methotrexate, adriamycin; to biological response modifiers, such as interferon and to toxins, such as ricin.

Methods of preparing and administering conjugates of the composite Hum4 V_L, V_H antibodies and a therapeutic agent are well known or readily determined. The pharmaceutical composition may be administered in a single dosage or multiple dosage form. Moreover, suitable dosages will depend on the age and weight of the patient and the therapeutic agent employed and are well known or readily determined.

Composite Hum4 V_L, V_H antibodies, and particularly composite Hum4 V_L, V_H single chain antibodies thereof, are particularly suitable for radioimmunoguided

surgery (RIGS). In RIGS, an antibody labeled with an imaging marker is injected into a patient having a tumor that expresses TAG-72. The antibody localizes to the tumor and is detected by a hand-held gamma detecting probe (GDP). The tumor is then excised (see Martin *et al.*
5   (1988), Amer. J. Surg., 156:386-392; and Martin *et al.* (1986), Hybridoma, 5:S97-S108). An exemplary GDP is the Neoprobe™ scanner, commercially available from Neoprobe Corporation, Columbus, OH. The relatively small size
10 and human character of the composite Hum4 V_L, V_H single chain antibodies will accelerate whole body clearance and thus reduce the waiting period after injection before surgery can be effectively initiated.

15   Administration and detection of the composite Hum4 V_L, V_H antibody-imaging marker conjugate may be accomplished by methods well-known or readily determined.

20   The dosage will vary depending upon the age and weight of the patient, but generally a one time dosage of about 0.1 to 200 mg of antibody-marker conjugate per patient is administered.

25

30

EXAMPLES

The following nonlimiting examples are merely for illustration of the construction and expression of composite Hum4 V_L, V_H antibodies. All temperatures not otherwise indicated are Centigrade. All percents not otherwise indicated are by weight.

Example I

CC49 and CC83 were isolated from their respective hybridomas using pNP9 as a probe (see Figure 5). CC49 V_H was obtained from p49 g1-2.3 (see Figure 6) and CC83 V_H was obtained from p83 g1-2.3 (see Figure 7), following the procedures set forth in EPO 0 365 997.

DNA encoding an antibody light chain was isolated from a sample of blood from a human following the protocol of Madisen et.al. (1987), Am. J. Med. Genet., 27:379-390) with several modifications. Two 5 ml purple-cap Vacutainer tubes (containing EDTA as an anticoagulant) were filled with blood and stored at ambient temperature for 2 hours. The samples were transferred to two 4.5 mL centrifuge tubes. To each tube was added 22.5 mL of filter-sterilized erythrocycte lysate buffer (0.155 M NH4Cl and 0.17 M Tris, pH 7.65, in a volume ratio of 9:1), and incubated at 37°C for 6.5 minutes. The tubes became dark red due to the lysed red blood cells. The samples were centrifuged at 9°C for 10 minutes, using an SS-34 rotor and a Sorvall centrifuge at 5,300 revolutions per minute (rpm) (~3,400 X g). The resulting white cell pellets were resuspended in 25 mL of 0.15 M NaCl solution. The white blood cells were then centrifuged as before. The pellets were resuspended in 500 µL of 0.15 M NaCl and transferred to

1.5 mL microcentrifuge tubes. The cells were pelleted again for 3 minutes, this time in the microcentrifuge at 3,000 rpm. Very few red blood cells remained on the pellet. After the supernatants were decanted from the two microcentrifuge tubes, 0.6 mL high TE buffer (100 mM
5   Tris, pH 8.0) was added. The tubes were hand-shaken for 10 and 15 minutes. The resulting viscous solution was extracted with phenol, phenol-chloroform and finally with just chloroform as described in Sambrook *et al.*,
10   *supra*. To 3.9 mL of pooled extracted DNA solution was added 0.4 mL NaOAc (3 M, pH 5), and 10 mL 100 percent ethanol. A white stringy precipitate was recovered with a yellow pipette tip, transferred into a new Eppendorf tube, washed once with 70 percent ethanol, and finally
15   washed with 100 percent ethanol. The DNA was dried *in vacuo* for 1 minute and dissolved in 0.75 mL deionized water. A 20 µL aliquot was diluted to 1.0 mL and the OD 260 nm value was measured and recorded. The concentration of DNA in the original solution was
20   calculated to be 0.30 mg/mL.

Oligonucleotides (oligos) were synthesized using phosphoramidite chemistry on a 380A DNA synthesizer (Applied Biosystems, Foster, CA) starting on
25   0.2 µM solid support columns. Protecting groups on the final products were removed by heating in concentrated ammonia solution at 55°C for 12 hours. Crude mixtures of oligonucleotides (approximately 12 OD 260 nm units)
30   were applied to 16 percent polyacrylamide-urea gels and electrophoresed. DNA in the gels was visualized by short wave UV light. Bands were cut out and the DNA eluted by heating the gel pieces to 65°C for 2 hours. Final purification was achieved by application of the eluted DNA solution onto C-18 Sep-Pac™ columns

(Millipore) and elution of the bound oligonucleotide
with a 60 percent methanol solution. The pure DNA was
dissolved in deionized distilled water (ddH$_2$O) and
quantitated by measuring OD 260 nm.

5   A GeneAmp™ DNA amplification kit (Cetus Corp.,
Emeryville, CA) was used to clone the Hum4 V$_L$ germline
gene by the PCR which was set up according to the
manufacturer's directions. A thermal cycler was used
for the denaturation (94 °C), annealing (45 °C) and
10   elongation (72 °C) steps. Each of the three steps in a
cycle were carried out for 4 minutes; there was a total
of 30 cycles.

      Upstream of the regulatory sequences in the
15 Hum4 V$_L$ germline gene, there is a unique *Cla* I
restriction enzyme site. Therefore, the 5' end
oligonucleotide for the PCR technique, called HUMVL(+)
(Figure 8), was designed to include this *Cla* I site.

20
      The 3' end oligonucleotide, called HUMVL(-)
(Figure 8), contained a unique *Hin*d III site; sufficient
mouse intron sequence past the splicing site to permit
an effective splice donor function; a human J4 sequence
25 contiguous with the 3' end of the V$_L$ exon of Hum4 V$_L$ to
complete the CDR3 and FR4 sequences of the V$_L$ domain
(see Figures 9 and 10); nucleotides to encode a tyrosine
residue at position 94 in CDR3; and 29 nucleotides close
to the 3' end of the V$_L$ exon of Hum4 V$_L$ (shown
30 underlined in the oligonucleotide HUMVL(-) in Figure 8)
to anneal with the human DNA target. In total, this 3'
end oligonucleotide for the PCR was 98 bases long with a
non-annealing segment (a "wagging tail") of 69
nucleotides. A schematic of the Hum4 V$_L$ gene target and

the oligonucleotides used for the PCR are shown in Figure 11.

A PCR reaction was set up with 1 μg of total human DNA in a reaction volume of 100 μL. Primers
5   HUMVL(-) and HUMVL(+) were each present at an initial concentratiuon of 100 pmol. Prior to the addition of *Taq* polymerase (2.5 units/reaction) 100 μLs of mineral oil were used to overlay the samples. Control samples were set up as outlined below. The samples were heated
10   to 95 °C for 3 minutes. When the PCR was complete, 20 μL samples were removed for analysis by agarose gel electrophoresis.

Based on the known size of the Hum4 V_L DNA
15   fragment to be cloned, and the size of the oligonucleotides used to target the gene, a product of 1099 bp was expected. A band corresponding to this size was obtained in the reaction (shown in lane 7, Figure
20   12).

To prepare a plasmid suitable for cloning and subsequently expressing the Hum4 V_L gene, the plasmid pSV2neo was obtained from ATCC and subsequently
25   modified. pSV2neo was modified as set forth below (see Figure 13).

The preparation of pSV2neo-101 was as follows. Ten micrograms of purified pSV2neo were digested with 40
30   units of *Hind* III at 37 °C for 1 hour. The linearized plasmid DNA was precipitated with ethanol, washed, dried and dissolved in 10 μL water. Two microliters each of 10 mM dATP, dCTP, dGTP and dTTP were added, as well as 2 μL of 10X ligase buffer. Five units (1 μL) of DNA polymerase I were added to make blunt the Hind III

sticky ends. The reaction mixture was incubated at room temperature for 30 minutes. The enzyme was inactivated by heating the mixture to 65°C for 15 minutes. The reaction mixture was phenol extracted and ethanol precipitated into a pellet. The pellet was dissolved in 20 µl deionized, distilled water. A 2 µl aliquot (ca. 1 µg) was then added to a standard 20 µL ligation reaction, and incubated overnight at 4 °C.

Competent *E.coli* DH1 cells were transformed with 1 µL and 10 µL aliquots of the ligation mix (Invitrogen, San Diego, CA) according to the manufacturer's directions. Ampicillin resistant colonies were obtained on LB plates containing 100 µg/mL ampicillin. Selected clones grown in 2.0 mL overnight cultures were prepared, samples of plasmid DNA were digested with *Hind* III and *Bam* HI separately, and a correct representative clone selected.

The resulting plasmid pSV2neo-101 was verified by size mapping and the lack of digestion with *Hind* III.

A sample of DNA from pSV2neo-10 mini-lysate was prepared by digesting with 50 units of *Bam* HI at 37°C for 2 hours. The linearized plasmid was purified from a 4 percent DNA polyacrylamide gel by electroelution. The DNA ends were made blunt by filling in the *Bam* HI site using dNTPs and Klenow fragment, as described earlier for the *Hind* III site of pSV2 neo-101.

A polylinker segment containing multiple cloning sites was incorporated at the *Bam* HI site of pSV2neo-101 to create pSV2neo-102. Equimolar amounts of two oligonucleotides, CH(+) and CH(-) (shown in Figure 14) were annealed by heating for 3 minutes at 90 °C and

cooling to 50 °C. Annealed linker DNA and blunt ended pSV2neo-101 were added, in a 40:1 molar volume to a standard 20 µL ligation reaction. *E.coli* DH1 was transformed with 0.5 µL and 5 µL aliquots of the ligation mixture (Invitrogen). Twelve ampicillin

5   resistant colonies were selected for analysis of plasmid DNA to determine whether the linker had been incorporated.

A *Hind* III digest of mini-lysate plasmid DNA

10 revealed linker incorporation in six of the clones. The plasmid DNA from several clones was sequenced, to determine the number of linker units that were blunt-end ligated to pSV2neo-101 as well as the relative orientation(s) with the linker. Clones for sequencing

15 were selected on the basis of positive digestion with *Hind* III.

A Sequenase™ sequencing kit (United States

20 B)iochemical Corp, Cleveland, OH was used to sequecne the DNA. A primer, NEO102SEQ, was used for sequencing and is shown in Figure 15. It is complementary to a sequence located upstream from the *Bam*HI site in the vector. Between 3 µg and 5 µg of plasmid DNA isolated

25 from *E.coli* mini-lysates were used for sequencing. The DNA was denatured and precipitated prior to annealing, as according to the manufacturer's instructions. Electrophoresis was carried out at 1500 volts; gels were dried prior to exposure to Kodak X-ray film. Data was

30 processed using Hitachi's DNASIS™ computer program.

From the DNA sequence data of 4 clones analyzed (see photograph of autoradiogram - Figure 16), compared to the expected sequence in Figure 14, two clones having

the desired orientationwere obtained. A representative clone was selected and designated pSV2neo-102.

A human Cк gene was inserted into pSV2neo-102 to form pRL1000. The human Cк DNA was contained in a

5   5.0 kb *Hind* III-*Bam* HI fragment (Hieter *et al.* (1980), <u>Cell</u>, 22:197-207).

A 3 µg sample of DNA from a mini-lysate of pSV2neo-102 was digested with *Bam* HI and *Hind* III. The

10   vector DNA was separated from the small *Bam* HI-*Hin*d III linker fragment, generated in the reaction, by electrophoresis on a 3.75 percent DNA polyacrylamide gel. The desired DNA fragment was recovered by electroelution. A pBR322 clone containing the 5.0 kb

15   *Hin*d III-*Bam* HI fragment of the human Cк gene (see Hieter *et al.*, *supra*) was designated phumCк. The 5.0 kb *Hin*d III-*Bam* HI fragment was ligated with pSV2neo-102r and introduced into *E.coli* DH1 (Invitrogen). Ampicillin

20   resistant colonies were screened and a clone containing the human Ck gene was designated pRL1000.

Finally, pRL1000 clones were screened by testing mini-lysate plasmid DNA from *E.coli* with *Hin*d III

25   and *Bam* HI. A clone producing a plasmid which gave 2 bands, one at 5.8 Kb (representing the vector) and the other at 5.0 kb (representing the human Cк insert) was selected. Further characterization of pRL1000 was achieved by sequencing downstream from the *Hin*d III site

30   in the intron region of the human Cк insert. The oligonucleotide used to prime the sequencing reaction was NEO102SEQ (Figure 15). Two hundred and seventeen bases were determined (see Figure 17). A new oligonucleotide corresponding to the (-) strand near the *Hin*d III site (shown in Figure 17) was synthesized so

that clones, containing the HHum4 V_L gene that were cloned into the *Cla* I and *Hind* III sites in pRL1000 (see Figure 13), could be sequenced.

5   A *Cla* I-*Hind* III DNA fragment containing Hum4 V_L obtained by PCR was cloned into the plasmid vector pRL1000. DNA of pRL1000 and the Hum4 V_L were treated with *Cla* I and *Hind* III and the fragments were gel purified by electrophoresis, as described earlier.

10   The pRL1000 DNA fragment and fragment containing Hum4 V_L gene were ligated, and the ligation mixture used to transform *E.coli* DH1 (Invitrogen), following the manufacturer's protocol. Ampicillin resistant clones were screened for the presence of the
15   Hum4 V_L gene by restriction enzyme analysis and a representative clone designated pRL1001 (shown in Figure 18).

Four plasmids having the correct *Cla* I-*Hind* III
20   restriction pattern were analyzed further by DNA sequencing of the insert region (see Figure 19). *Hind* III Cκ(-) (shown in Figure 17), HUMLIN1(-) (shown in Figure 10), HUMLIN2(-) (shown in Figure 10) were used as
25   the sequencing primers. Two out of the four plasmids analyzed had the expected sequence in the coding regions (Figure 19, clones 2 and 9).

Clone 2 was chosen and used for generating
30   sufficient plasmid DNA for cell transformations and other analysis. This plasmid was used for sequencing through the Hum4 V_L, and the upstream region to the *Cla* I site. Only one change at nucleotide position 83 from a C to a G (Figure 10) was observed, compared to a

published sequence (Klobeck *et al.* (1985), supra). The DNA sequence data also indicates that the oligonucleotides used for the PCR had been correctly incorporated in the target sequence.

5   The Biorad Gene Pulser™ apparatus was used to transfect Sp2/0 cells with linearized plasmid DNAs containing the light or heavy chain constructs. The Hum4 V_L was introduced in Sp2/0 cells along with corresponding heavy chains by the co-transfection scheme
10   indicated in Table 1.

Table 1

Cell Line Designation	DNA Added		
	L Chain pRL1001	H Chain p49 g1-2.3	H Chain p83 g1-2.3
MP1-44H	20 µg	15 µg	0 µg
MP1-84H	20 µg	0 µg	15 ug

A total of 8.0×10^6 Sp2/0 cells were washed in sterile PBS buffer (0.8 mL of 1 X 107 viable cells/mL) and held on ice for 10 minutes. DNA of pRL1001,
25   linearized at the *Cla* I site, and the DNA of either p49 g1-2.3 or p83 g1-2.3, linearized at their respective *Nde* I sites, were added, in sterile PBS, to the cells (see protocol - Table 2) and held at 0 °C for a further 10 minutes. A single 200 volt, 960 µF electrical pulse
30   lasting between 20 and 30 milliseconds was used for the electroporation. After holding the perturbed cells on ice for 5 minutes, 25 mL of RPMI medium with 10 percent fetal calf serum were introduced, and 1.0 mL samples aliquoted in a 24 well tissue culture plate. The cells were incubated at 37 °C in a 5 percent CO_2 atmosphere.

After 48 hours, the media was exchanged with fresh
selection media, now containing both 1 mg/mL Geneticin
(G418) (Difco) and 0.3 μg/ml mycophenolic acid/gpt
medium. Resistant cells were cultured for 7-10 days.

5   Supernatants from wells having drug resistant
colonies were tested on ELISA plates for activity
against TAG-72. A roughly 10 percent pure TAG-72
solution prepared from LS147T tumor xenograft cells was
diluted 1:40 and used to coat flexible polyvinyl
10 chloride microtitration plates (Dynatech Laboratories,
Inc.). Wells were air-dried overnight, and blocked the
next day with 1 percent BSA. Supernatant samples to be
tested for anti-TAG-72 antibody were added to the washed
15 wells and incubated for between 1 and 2 hours at 37 °C.
Alkaline phosphatase labeled goat anti-human IgG
(diluted 1:250) (Southern Biotech Associates,
Birmingham, AL) was used as the probe antibody.
Incubation was for 1 hour. The substrate used was p-
20 nitrophenylphosphate. Color development was terminated
by the addition of 1.0 N NaOH. The plates were read
spectrophotometrically at 405 nm and 450 nm, and the
values obtained were 405 nm-450 nm.

25   Those samples producing high values in the
assay were subcloned from the original 24 well plate
onto 96 well plates. Plating was done at a cell density
of half a cell per well (nominally 50 cells) to get pure
monoclonal cell lines. Antibody producing cell lines
30 were frozen down in media containing 10 percent DMSO.

Two cell lines were procured having the
designations: MP1-44H and MP1-84H.   MP1-44H has the
chimeric CC49 γ1 heavy chain with the Hum4 V_L light

chain; and MP1-84H has the chimeric CC83 g1 heavy chain with the HumVkIV light chain.

A 1.0 L spinner culture of the cell line MP1-44H was grown at 37°C for 5 days for antibody production. The culture supernatant was obtained free of cells by centrifugation and filtration through a 0.22 micron filter apparatus. The clarified supernatant was passed over a Protein A cartridge (Nygene, New York). Immunoglobulin was eluted using 0.1 M sodium citrate buffer pH 30. The pH of the eluting fractions containing the antibody was raised to neutrality by the addition of Tris base, pH 9.0. The antibody-containing fractions were concentrated and passed over a Pharmacia Superose 12 HR 10/30 gel filtration column. A protein was judged to be homogeneous by SDS polyacrylamide gel electrophoresis. Isoelectric focusing further demonstrated the purity of MP1-44H.

The biological performance of the human composite antibody, MP1-44H, was evaluated by comparing immunohistochemistry results with two other anti-TAG-72 antibdoies CC49 (ATCC No. HB 9459) and Ch44 (ATCC No. HB 9884). Sections of human colorectal tumor embedded in paraffin were tested with the three antibodies by methods familiar to those skilled in this art. All three antibodies gave roughly equivalent binding recognition of the tumor antigen present on the tumor tissue sample.

A further test of the affinity and biological integrity of the human composite antibody MP1-44H was a competition assay, based on cross-competing radioiodine--labeled versions of the antibody with CC49 and Ch44 in all combinations. From the data shown in Figure 20, it

is apparent that the affinity of all 3 antibodies is equivalent and can bind effectively to tumor antigen.

MP1-44H (ATCC HB 10426) and MP1-84H (ATCC HB 10427) were deposited at the American Type Culture

5 Collection (ATCC). The contract with ATCC provides for permanent availability of the cell lines to the public on the issuance of the U.S. patent describing and identifying the deposit or the publications or upon the laying open to the public of any U.S. or foreign patent

10 application, which ever comes first, and for availability of the cell line to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 CFR §122 and the Commissioner's rules pursuant thereto (including 37 CFR

15 §1.14 with particular reference to 886 OG 638). The assignee of the present application has agreed that if the cell lines on deposit should die or be lost or destroyed when cultivated under suitable conditions for

20 a period of thirty (30) years or five (5) years after the last request, it will be promptly replaced on notification with viable replacement cell lines.

Example 2

25

Single-chain antibodies consist of a V_L, V_H and a peptide linker joining the V_L and V_H domains to produce SCFVs. A single chain antibody, SCFV1, was constructed to have the Hum4 V_L as V Domain 1 and CC49

30 V_H as V Domain 2 (see Figure 21).

The polypeptide linker which joins the two V domains was encoded by the DNA introduced at the 3' end of the V_L DNA during the PCR. The oligonucleotides SCFV1a and SCFV2 were designed to obtain the DNA segment

incorporating part of the yeast invertase leader
sequence, the Hum4 V_L and the SCFV linker.

The polypeptide linker for SCFV1 was encoded in
oligonucleotide SCFV1b (see below). The underlined
5   portions of the oligonucleotides SCFV1a and SCFV1b are
complementary to sequences in the Hum4 V_L and linker
respectively. The sequences of SCFV1a and SCFV1b are as
follows, with the hybridizing sequences underlined:

10

SCFV1a with the *Hind* III in bold:

Hind III
.15
5'CTGCAAGCTTCCTTTTCCTTTTGGCTGGTTTTGCAGCCAAAATATCTGCAG
ACATCGTGATGACCCAGTC-3'

SCFV1b with the *Aat* II site in bold:
20

5'-CGTAAGAC<u>GTCTAAGGAACGAAATTGGGCCAATTGTTCTGAGGA</u>
<u>GACCGAACCTGACTCCTT</u>CACCTTGGTCCCTCCGCCG-3'

25

The target DNA in the PCR was pRL1001
(shown in Figure 18). The PCR was performed pursuant to
the teachings of Mullis *et al.* *supra*. A DNA fragment
30   containing the Hum4 V_L-linker DNA component for the
construction of SCFV1 was obtained and purified by
polyacrylamide gel electrophoresis according to the
teachings of Sambrook *et al.*, *supra*.

p49 g1-2.3, containing CC49 V$_H$, was the target DNA in the PCR. PCR was performed according to the methods of Mullis *et al.*, *supra*. The oligonucleotides used for the PCR of CC49 V$_H$ are as follows, with the hybridizing sequences underlined:

5   SCFV1c, with the *Aat* II site in bold:

10

5'-CCTTAGACGTCCAGTTGCAGCAGTCTGACGC-3'

SCFV1d, with the *Hin*d III site in bold:
15

20   5'-GATCAAGCTTCACTAGGAGACGGTGACTGAGGTTCC-3'

The purified Hum4 V$_L$-linker and V$_H$ DNA fragments were treated with *Aat* II (New England Biolabs,
25   Beverly, MA) according to the manufacturer's protocol, and purified from a 5 percent polyacrylamide gel after electrophoresis. An equimolar mixture of the *Aat* II fragments was ligated overnight. The T4 DNA ligase was heat inactivated by heating the ligation reaction
30   mixture at 65 °C for 10 minutes. Sodium chloride was added to the mixture to give a final concentration of 50 mM and the mixture was further with *Hin*d III. A *Hin*d III DNA fragment was isolated and purified from a 4.5 percent polyacrylamide gel and cloned into a yeast expression vector (see Carter *et al.* (1987), In: DNA

Cloning, A Practical Approach, Glover (ed.) Vol. III:
141-161). The sequence of the fragment, containing the
contiguous SCFV1 construct, is set forth in Figure 22.

The anti-TAG-72 SCFV1 described herein
5 utilized the yeast invertase leader sequence (shown as
positions -19 to -1 of Figure 22), the Hum4 V_L (shown as
positions 1 to 113 of Figure 22), an 18 amino acid
linker (shown as positions 114 to 132 of Figure 22) and
CC49 V_H (shown as positions 133 to 248 of Figure 22).
10

The complete DNA and amino acid sequence of
SCFV1 is given in Figure 22. The oligonucleotides used
to sequence the SCFV1 are set forth below.

15 TPI:

5'-CAATTTTTTGTTTGTATTCTTTTC-3'.

HUVKF3:

20 5'-CCTGACCGATTCAGTGGCAG-3'.

DC113:

5'-TCCAATCCATTCCAGGCCCTGTTCAGG-3'.

25
SUC2T:

5'-CTTGAACAAAGTGATAAGTC-3'.

Example 3
30

A plasmid, pCGS517 (Figure 23), containing
a prorennin gene was digested with Hind III and a 6.5 kb
fragment was isolated. The plasmid pCGS517 has a
triosephosphate isomerase promoter, invertase [SUC2]
signal sequence, the prorennin gene and a [SUC2]

terminator. The *Hind* III-digested SCFV1 insert obtained above (see Figure 23) was ligated overnight with the *Hind* III fragment of pCGS517 using T4 DNA ligase (Stratagene, La Jolla, CA).

5   The correct orientation existed when the *Hind* III site of the insert containing part of the invertase signal sequence ligated to the vector DNA to form a gene with a contiguous signal sequence. *E.coli* DHI (Invitrogen) cells were transformed and colonies

10   screened using a filter-microwave technique (see Buluwela, *et al.* (1989), Nucleic Acids Research, 17:452). From a transformation plate having several hundred colonies, 3 positive clones were obtained. Digesting the candidate plasmids with *Sal* I and *Kpn* I, each a

15   single cutter, differentiated between orientations by the size of the DNA fragments produced. A single clone, pDYSCFV1 (Figure 23), had the correct orientation and was used for further experimentation and cloning. The

20   probe used was derived from pRL1001, which had been digested with *Kpn* I and *Cla* I (see Figure 18). The probe DNA was labeled with ^{32}P α-dCTP using a random oligonucleotide primer labeling kit (Pharmacia LKB Biotechnology, Piscataway, NJ).

25

          The next step was to introduce the *Bgl* II-*Sal* 1 fragment from pDYSCFV1 into the same restriction sites of another vector (ca. 9 kb), which was derived from PCGS515 (Figure 23). to give an autonomously

30   replicating plasmid in *S.cerevisiae*.

          DNA from the vector and insert were digested in separate reactions with *Bgl* II and *Sal* I using 10X buffer number 3 (50 MM Tris-HCI (pH 8.0), 100 mM NaCl, BRL). The DNA fragment from pDYSCFV1 was run

in and electroeluted from a 5 percent polyacrylamide gel
and the insert DNA was run and electroeluted from a 3.75
percent polyacrylamide gel. A standard ligation using
T4 DNA ligase (Stratagene) and a transformation using *E.
coli* DH1 (Invitrogen) was carried out. Out of 6 clones
selected for screening with *Bgl* II and *Sal* II, all 6
were correctly oriented, and one was designated
pCGS515/SCFV1 (Figure 23).

DNA sequencing of pCGS515/SCFVI DNA was
done using a Sequenase™ kit (U.S. Biochemical,
Cleveland, OH) using pCGS515/SCFV1 DNA. The results
have been presented in Figure 22 and confirm the
sequence expected, based on the linker, the Hum4 V_L and
the CC49 V_H.

Transformation of yeast cells using the
autonomosly replicating plasmid pCGS515/SCFV1 was
carried out using the lithium acetate procedures
described in Ito *et al.* (1983), J. Bacteriol., 153:163-
168; and Treco (1987), In: Curent Protocols in Molecular
Biology, Ausebel *et al.* (eds), 2:13.71-13.7.6. The
recipient strain of *S.cerevisiae* was CGY1284 having the
genotype - MAT α (mating strain α), ura 3-52 (uracil
auxotrophy), SSC1-1 (supersecreting 1), and PEP4+
(peptidase 4 positive).

Transformed clones of CGY1284 carrying SCFV
plasmids were selected by their ability to grow on
minimal media in the absence of uracil. Transformed
colonies appeared within 3 to 5 days. The colonies were
transferred, grown and plated in YEPD medium. Shake
flasks were used to provide culture supernatant with
expressed product.

An ELISA procedure was used to detect
biological activity of the SCFV1. The assay was set up
such that the SCFV would compete with biotinylated CC49
(biotin-CC49) for binding to the TAG-72 antigen on the
ELISA plate .

5

SCFV1 protein was partially purified from a
crude yeast culture supernatant, using a Superose 12 gel
filtration column (Pharmacia LKB Biotechnology), and
found to compete with biotinylated CC49 in the
competition ELISA. These results demonstrate that the
SCFV1 had TAG-72 binding activity.

10

The SCFV1 protein has been detected by a
standard Western protocol (see Towbin *et al.* (1979), Proc.
Natl. Acad. Sci., U.S.A., 76:4350-4354). The detecting
agent was biotinylated FAID14 (ATCC No. CRL 10256), an
anti-idiotypic monoclonal antibody prepared from mice
that had been immunized with CC49. A band was
visualized that had an apparent molecular weight of
approximately 26,000 daltons, the expected size of the
SCFV1. This result demonstrated that the SCFV1 had been
secreted and properly processed.

15

20

25 Example 4

The following example demonstrates the cloning
of human V_H genes into a SCFV plasmid construct
containing sequence coding for the Hum4 V_L and a 25
amino acid linker called UNIHOPE.

30

A vector was prepared from plasmid pRW 83
containing a chloramphenicol resistance (Cam^r) gene for
clone selection, and a *penP* gene with a *penP* promoter
and terminator (see Mezes, *et al.* (1983), J. Biol. Chem.,
258:11211-11218) and the *pel B* signal sequence (see Lei

et al. (1987) *supra*). The vector was designated Fragment
A. (see Figure 24). The *penP* gene was removed with a
Hind III/*Sal* I digest.

5   The *penP* promoter and *pel B* signal sequence were
obtained by a PCR using pRW 83 as a template and
oligonucleotides penP1 and penP2 as primers. The
fragment was designated Fragment B (see Figure 24). A
Nco I enzyme restriction site was introduced at the 3'
10 end of the signal sequence region by the penP2
oligonucleotide.

penP1:

5'-CGATAAGCTTGAATTCCATCACTTCC-3'

15 penP2:

5'-GGCCATGGCTGGTTGGGCAGCGAGTAATAACAATCCAGCG GCT
GCCGTAGGCAATAGGTATTTCATCAAAATCGTCTCCCTCCGTTTGAA-3'

A SCFV comprised of a Hum4 V_L, a CC49 V_H, and
20 an 18 amino acid linker (Lys Glu Ser Gly Ser Val Ser Ser
Glu Gln Leu Ala Gln Phe Arg Ser Leu Asp) was obtained
from pCGS515/SCFV1 by PCR using oligonucleotides penP3
and penP6. This fragment was designated Fragment D (see
Figure 24). A *Bcl* I site was introduced at the 3' end
25 of the V_H region by the penP6 oligonucleotide.

penP3:

5'-GCTGCCCAACCAGCCATGGCCGACATCGTGATGACCCAGTCTCC-3'

30 penP6(-):

5'-CTCTTGATCACCAAGTGACTTTATGTAAGATGATGTTTTG ACG
GATTCATCGCAATGTTTTTATTTGCCGGAGACGGTGACTGAGGTTCC-3'

Fragments B and D were joined by PCR using
oligonucleotides penP1 and penP6, following the

procedures of Horton *et al.*, *supra*. The new fragment was designated E (See Figure 24).

Fragment C containing the *penP* termination codon was isolated by digesting pRW 83 with *Bcl* I and *Sal* I, and designated Fragment C. pRW 83 was isolated from *E. coli* strain GM161, which is DNA methylase minus or dam⁻.

Plasmid pSCFV 31 (see Figure 24) was created with a three part ligation Fragments A, C, and E.

The *Nco* I restriction enzyme site within the Camʳ gene and the *Hind* III site located at the 5' end of the *penP* promoter in pSCFV 31 were destroyed through a PCR DNA amplification using oligonucleotides Nco1.1 and Nco1.3(-) to generate an *Eco* RI-*Nco* I fragment and oligonucleotides Nco1.2 and Nco1.4c(-) to generate a *Nco* I to *Eco* RI fragment. These two fragments were joined by PCR-SOE using oligonucleotides Nco1.1 and Nco1.4c(-). The oligonucleotides are set forth below:

Nco1.1:

5'-TCCGGAATTCCGTATGGCAATGA-3'

Nco1.3(-):
5'-CTTGCGTATAATATTTGCCCATCGTGAAAACGGGGGC-3'

Nco1.2:

5'-ATGGGCAAATATTATACGCAAG-3'

Nco1.4c(-):

5'-CACTGAATTCATCGATGATAAGCTGTCAAACATGAG-3'

pSCFV 31 was digested with *Eco* RI and the larger fragment was isolated by polyacrylamide gel electrophoresis. To prevent self ligation, the DNA was

dephosphorylated using calf intertinal alkaline phosphatase according to the teachings of Sambrook *et al.*, *supra*.

A two part ligation of the larger pSCFV 31 digested fragment and the PCR-SOE fragment, described above, resulted in the creation of pSCFV 31b (see Figure 25).

pSCFV 31b was digested with *Nco* I and *Sal* I and a fragment containing the Cam^r gene was isolated.

The Hum4 V$_L$ was obtained by PCR DNA amplification using pCGS515/SCFV1 as a template and oligonucleotides 104BH1 and 104BH2(-) as primers.

104BH1:

5'-CAGCCATGGCCGACATCGTGATGACCCAGTCTCCA-3'

104BH2(-):

5'-AAGCTTGCCCCATGCTGCTTTAACGTTAGTTTTATCTGCTGG AGACAGAGTGCCTTCTGCCTCCACCTTGGTCCCTCCGCCGAAAG-3'

The CC49 V$_H$ was obtained by PCR using p49 g1-2.3 (Figure 5) as a template and oligonucleotides 104B3 and 104B4(-) as primers. A *Nhe* I enzyme restriction site was introduced just past the termination codon in the 3' end (before the *Bcl* I site) by oligonucleotide 104B4(-).

104B3:

5'-GTTAAAGCAGCATGGGGCAAGCTTATGACTCAGTTGCAGCAGTCTGACGC-3'

104B4(-):

5'-CTCTTGATCACCAAGTGACTTTATGTAAGATGATGTTTTGACGGATT
CATCGCTAGCTTTTTATTTGCCATAATAAGGGGAGACGGTGACTGAGGTTCC-3'

5   In the PCR which joined these two fragments
using oligonucleotides 104BH1 and 104B4(-) as primers, a
coding region for a 22 amino acid linker was formed.

         A fragment C (same as above) containing the
10 penP termination codon was isolated from pRW 83 digested
with Bcl I and Sal I.

         Plasmid pSCFV 33H (see figure 25) was
created with a three part ligation of the vector,
15 fragment C, and the SCFV fragment described above.

         pSCFV 33H was digested with NcoI and NheI,
and the DNA fragment containing the Cam^r gene was
isolated as a vector.

20
         Hum4 V$_L$ was obtained by PCR DNA
amplification using pRL1001 (see Figure 18) as a
template and oligonucleotides UNIH1 and UNIH2(-) as
primers. Oligonucleotides for the PCR were:

25
UNIH1:

5'-CAGCCATGGCCGACATTGTGATGTCACAGTCTCC-3'

The Nco I site is in bold and the hybridizing sequence
30 is underlined.

UNIH2(-):

5'-GAGGTCCGTAAGATCTGCCTCGCTACCTAGCAAA
AGGTCCTCAAGCTTGATCACCACCTTGGTCCCTCCGC-3'

The Hind III site is in bold.

The CC49 V_H was obtained by a PCR using p49g1-2.3 (see Figure 6) as a template and oligonucleotides UNI3 and UNI4(-) as primers.

UNI3:

5'-AGCGAGGCAGATCTTACGGACCTCGAG<u>GTTCAGTTGCAGCAGTCTGAC</u>-3'.

The *Xho* I site is in bold and the hybridizing sequence is underlined.

UNI4(-):

5'-CATCGCTAGC<u>TTTTTATGAGGAGACGGTGACTGAGGTTCC</u>-3'.

The *Nhe* I site is in bold and the hybridizing sequence is underlined.

Oligonucleotides UNIH1 and UNI4(-) were used in the PCR-SOE amplification which joined the Hum4 V_L and CC49 V_H fragments and formed a coding region for a negatively charged fifteen amino acid linker. The DNA was digested with *Nhe* I and *Nco* I and ligated with the vector fragment from the *Nco* I-*Nhe* I digest of pSCFV 33H. The resultant plasmid was designated pSCFV UNIH (shown in Figure 25).

With the construction of pSCFV UNIH, a universal vector for any SCFV was created with all the desired restriction enzyme sites in place.

pSCFV UNIH was digested with *Hind* III/*Xho* I, and the large DNA fragment containing the Cam^r gene, Hum4 V_L and CC49 V_H was isolated.

A fragment coding for a 25 amino acid linker, was made by annealing the two oligonucleotides shown below. The linker UNIHOPE is based on 205C SCA™ linker (see Whitlow, (1990) <u>Antibody Engineering: New Technology and Application Implications</u>. IBC USA

Conferences Inc, MA), but the first amino acid was changed from serine to leucine and the twenty-fifth amino acid were was changed from glycine to leucine, to accomodate the *Hind* III and *Xho* I restriction sites. The nucleotide sequence encoding the linker UNIHOPE is set forth below:

UNIHOPE (Figure 26):

5'-TATAAAGCTTAGTGCGGACGATGCGAAAAAGGATGCTGCGAAG AAGGATGACGCTAAGAAAGACGATGCTAAAAAGGACCTCGAGTCTA-3'

UNIHOPE(-) (Figure 26):

5'TAGACTCGAGGTCCTTTTTAGCATCGTCTTTCTTAGCGT CAT CCTTCTTCGCAGCATCCTTTTTCGCATCGTCCGCACTAAGCTTTATA-3'

The resulting strand was digested with *Hind* III/*Xho* I and ligated into the vector, thus generating the plasmid pSCFV UHH (shown in Figure 27). Plasmid pSCFV UHH expresses a biologically active, TAG-72 binding SCFV consisting of the Hum4 V_L and CC49 V_H. The expression plasmid utilizes the β-lactamase *penP* promoter, pectate lyase *pelB* signal sequence and the *penP* terminator region. Different immunoglobulin light chain variable regions can be inserted in the *Nco* I-*Hind* III restriction sites, different SCFV linkers can be inserted in the *Hind* III-*Xho* I sites and different immunoglobulin heavy chain variable regions can be inserted in the *Xho* I-*Nhe* I sites.

E.coli AG1 (Stratagene) was transformed with the ligation mix, and after screening, a single chloramphenicol resistant clone, having DNA with the correct restriction map, was used for further work.

The DNA sequence and deduced amino acid sequence of the SCFV gene in the resulting plasmid are shown in Figure 26.

E.coli AG1 containing pSCFV UHH were grown in 2 ml of LB broth with 20 µg/mL chloramphenicol (CAM 20). The culture was sonicated and assayed using a competition ELISA. The cells were found to produce
5   anti-TAG-72 binding material. The competition assay was set up as follows: a 96 well plate was derivatized with a TAG-72 preparation from LS174T cells. The plate was blocked with 1% BSA in PBS for 1 hour at 31 °C and then washed 3 times with 200 µL of biotinylated CC49
10   (1/20,000 dilution of a 1 mg/mL solution) were added to the wells and the plate was incubated for 30 minutes at 31 °C. The relative amounts of TAG-72 bound to the plate, biotinylated CC49, streptavidin-alkaline phosphatase, and color development times were determined
15   empirically in order not to have excess of either antigen or biotinylated CC49, yet have enough signal to detect competition by SCFV. Positive controls were CC49 at 5 µg/mL and CC49 Fab at 10 µL/mL. Negative controls
20   were 1% BSA in PBS and/or concentrated LB. Unbound proteins were washed away.

Fifty microliters of a 1:1000 dilution of streptavidin conjugated with alkaline phosphatase (Southern Biotechnology Associates, Inc., Birmingham,
25   AL) were added and the plate was incubated for 30 minutes at 31 °C. The plate was washed 3 more times. Fifty microliters of a para-nitrophenylphosphate solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) were added and the color reaction was
30   allowed to develop for a minimum of 20 minutes. The relative amount of SCFV binding was measured by optical density scanning at 405-450 nm using a microplate reader (Molecular Devices Corporation, Menlo Park, CA). Binding of the SCFV resulted in decreased binding of the

biotinylated CC49 with a concomitant decrease in color development. The average value for triplicate test samples is shown in the table below:

Sample (50 µL) (mixed 1:1 with CC49 Biotin)	OD 405 nm – OD 450 nm Value at 50 minutes
Sonicate *E.coli* AG1/ pSCFVUHH clone 10	0.072
Sonicate *E.coli* AG1/ pSCFVUHH clone 11	0.085
CC49 at 5 mg/mL	0.076
CC49 Fab at 10 mg/mL	0.078
LB (negative control)	0.359

20   The data indicates that there was anti-TAG-72 activity present in the *E.coli* AGI/pSCFVUHH clone sonicate.

Example 7

25   The plasmid pSCFVUHH may be used to host other V_H genes on *Xho* I-*Nhe* I fragments and test in a SCFV format, following the procedures set forth below. A schematic for this process is shown here.

30

Discovery of Hum4 V_L-V_H combinations that compete with known prototype
TAG-binding antibodies or mimetics.

pSCFVUHH *Xho* I/*Nhe* I
Vector DNA Fragment
(CC49 V_H removed)
or pATDFLAG XhoI/NheI Vector DNA Fragment

Isolate mRNA from
peripheral blood lymphocytes

↓

Synthesize cDNA

↓

PCR amplify human V_H genes
using oligos HVH135, HVH2A,
HVH46 (as the 5' targeting
oligos) and JH1245, JH3 and
JH6 (as the 3' targeting oligos)
in all 9 combinations.

↓

Gel purify DNA

↓

Digest with *Xho* I and *Nhe* I

↓

Gel purify DNA (V_H inserts)

Ligate Vector
and
V_H insert DNAs   Transform
                                      E.coli

-62-

Plate transformation mix onto hydrophilic membranes
(137 mm) which are placed on LB CAM 20 agar plates
(150 mm) with a colony density of $\leq$ 50,000 per plate.
Grow for 8-16 hours at 37 °C.

SCFV is
secreted
by *E.coli*
and may
bind to TAG.

Transfer hydrophilic membrane onto fresh LB CAM 20 plate
having a TAG-72-coated hydrophobic membrane (137 mm) already
placed on the agar surface. Incubate for 24-96 hours.

assay

Process hydrophobic membrane using a prototype
biotinylated TAG-competing antibody, e.g. B72.3, CC49,
CC83 or biotinylated competing peptide or mimetic. Use
streptavidin conjugated with alkaline phosphatase to bind
to biotin and suitable substrate for alkaline phosphatase to
develop a color reaction.

Co-relate clear zones on membrane assay with colony(ies)
on hydrophilic membrane. Isolate/purify correct clone
as necessary. Characterize DNA (sequence) and determine
binding affinity of SCFV to TAG-72. Purify SCFV and
perform *in vivo* animal biodistribution studies.

Determine normal:tumor tissue binding profile by
immunohistochemistry.

Utilize Hum4 V_L and V_H in preferred antibody
formats e.g. whole Ig (IgG1, IgE, IgM etc.) Fab or
F(ab')$_2$ fragment, or SCFV.

Isolating total RNA from peripheral blood lymphocytes:

Blood from a normal, healthy donor is drawn into three 5 mL purple-cap Vacutainer tubes. Seven mL of blood are added to two 15 mL polypropylene tubes. An

5 equal volume of lymphoprep (cat# AN5501, Accurate) is added and the solution is mixed by inversion. Both tubes are centrifuged at 1000 rpm and 18 °C for 20 minutes. The resulting white area near the top of the liquid (area not containing red blood cells) is removed

10 from each sample and placed into two sterile polypropylene centrifuge tube. Ten mL of sterile PBS are added and the tube mixed by inversion. The samples are centrifuged at 1500 rpm and 18 °C for 20 minutes

15 Total RNA is isolated from resulting pellet according to the RNAzol B Method (Chomczynski and Sacchi (1987), _Analytical Biochemistry_, 162:156-159). Briefly, the cell pellets are lysed in 0.4 mL RNAzol solution (cat#:CS-105, Cinna/Biotecx). RNA is solubilized by

20 passing the cell pellet through a 1 mL pipet tip. Sixty μL of chloroform are added and the solution is shaken for 15 seconds. RNA solutions are then placed on ice for 5 minutes. Phases are separated by centrifugation at 12000 x g and 4 °C for 15 minutes. The upper

25 (aqueous) phases are transferred to fresh RNase-free microcentrifuge tubes. One volume of isopropanol is added and the samples placed at -20 °C for 1 hour. The samples are then placed on dry ice for 5 minutes and

30 finally centrifuged for 40 seconds at 14,000 x g and 4 °C. The resulting supernatant is removed from each sample and the pellet is dissolved in 144 μL of sterile RNase-free water. Final molarity is brought to 0.2M NaCL. The DNA is reprecipitated by adding 2 volumes of 100% ethanol, leaving on dry ice for 10 minutes, and

centrifugation at 14,000 rpm and 4 °C for 15 minutes.
The supernatants are then removed, the pellets washed
with 75% ethanol and centrifuged for 8 minutes at 12000
x g and 4 °C. The ethanol is then removed and the
pellets dried under vacuum. The resulting RNA is then
5 dissolved in 20 sterile water containing 1 µl RNasin
(cat#:N2511, Promega).

cDNA synthesis:

cDNA synthesis is performed using a Gene Amp™
10 PCR kit (cat#: N808-0017 Perkin Elmer Cetus), RNasin™
(cat#: N2511, Promega), and AMV reverse transcriptase
(cat#: M9004, Promega). The following protocol is used
for each sample:

Components	Amount
MgCl$_2$ solution	4 µl
10 µl PCR buffer II	2 µl
dATP	2 µl
dCTP	2 µl
dGTP	2 µl
dTTP	2 µl
3' primer (random hexamers)	1 µl
RNA sample	2 µl
RNasin	1 µl
AMV RT	1.5 µl

Samples are heated at 80 °C for 3 minutes then
slowly cooled to 48 °C. The samples are then
centrifuged for 10 seconds. AMV reverse transcriptase
is added to the samples which are then incubated for 30
minutes at 37 °C. After incubation. 0.5 µl of each dNTP

and 0.75 reverse transcriptase (cat#:109118, Boehringer
Mannheim) are added. The samples are incubated for an
additional 15 minutes at 37 °C.

<u>PCR Reaction</u>:

5   Oligonucleotides are designed to amplify human
V_H genes by polymerase chain reaction. The 5'
oligonucleotides are set forth below:

HVH 135:

10 5'-TATTCTCGAGGTGCA(AG)CTG(CG)TG(CG)AGTCTGG-3'

HVH2A:

5'-TATTCTCGAGGTCAA(CG)TT(AG)A(AG)GGAGTCTGG-3'

HVH46:

15 5'-TATTCTCGAGGTACAGCT(AG)CAG(CG)(AT)GTC(ACG)GG-3'

The 3' oligonucleotides are set forth below:

JH1245:

5'-TTATGCTAGCTGAGGAGAC(AG)GTGACCAGGG-3'

20 JH3:

5'-TTATGCTAGCTGAAGAGACGGTGACCATTG

JH6:

5'-TTATGCTAGCTGAGGAGACGGTGACCGTGG-3'

25   PCR reactions are performed with a GeneAmp™ PCR
kit (cat#:N808-0017, Perkin Elmer Cetus). Components
are listed below:

30

Components	Amount
ddH$_2$O	75 µl
10 x buffer	10 µl
dATP	2 µl
dCTP	2 µl
dGTP	2 µl
dTTP	2 µl
1* Target DNA	1 µl
2* 5' primer	2.5 µl
3' primer	2.0 µl
3* AmpliTaq™ Polymerase	1.3 µl

*components added in order at 92 °C of first cycle

PCR program: step 1   94 °C for 30 seconds

step 2   60 °C for 1 minutes

step 3   72 °C for 45 seconds

Approximately 35 cycles are completed for each reaction. All PCR reactions are performed using a Perkin Elmer Cetus PCR System 9600 thermal cycler.

Treatment of Human V$_H$ inserts with *Xho* I and *Nhe* I:

Human V$_H$ genes are digested with *Xho* I (cat#: 131L, New England Biolabs) and *Nhe* I (cat#: 146L, New England Biolabs). The following protocol is used for each sample:

SUBSTANCE	AMOUNT
DNA	20 µl
NEB Buffer #2	4.5 µl
Nhe I	2 µl
Xho I	2 µl
ddH$_2$O	16.5 µl

Samples are incubated at 37 °C for one hour. After this incubation, an additional 1.5 µL *Nhe* I is added and samples are incubated an additional two hours at 37 °C.

Purification of DNA:

After the restrictive enzyme digest, DNA is run on a 5 percent polyacrylamide gel (Sambrook *et al.* (1989), *supra*). Bands of 390-420 bp in size are excised from the gel. DNA is electroeluted and ethanol precipitated according to standard procedures.

PCR products resulting from oligonucleotide combinations are pooled together: JH1245 with HVH135, HVH2A and HVH46; JH3 with HVH135, HVH2A and HVH46; JH6 with HVH135, HVH2A and HVH46. The volume of the resulting pools are reduced under vacuum to 50 microliters. The pools are then purified from a 4 percent polyacrylamide gel (Sambrook *et al.* (1989), *supra*) to isolate DNA fragments. Bands resulting at 390-420 bp are excised from the gel. The DNA from excised gel slices is electroeluted according to standard protocols set forth in Sambrook, *supra*.

Isolation of pSCFVUHH *Xho* I/*Nhe* I Vector Fragment

Approximately 5 µg in 15 µL of pSCFVUHH plasmid
is isolated using the Magic Mini-prep™ system
(Promega). To this is added 5.4 µL OF 10X Buffer #2

5   (New England Biolabs), 45 units of *Xho* I (New England
Biolabs), 15 units of *Nhe* I and 24 µL of ddH2O. The
reaction is allowed to proceed for 1 hour at 37 °C. The
sample is loaded on a 4% polyacrylamide gel,
electrophoresed and purified by electroelution, as

10 described earlier. The DNA pellet is dissolved in 20 µL
of ddH2O.

One hundred nanograms of pSCFVUHH digested with
Xho I/*Nhe* I is ligated with a 1:1 molar ratio of

15 purified human VH inserts digested with *Xho* I and *Nhe* I
using T4 DNA ligase (Stratagene). Aliquots are used to
transform competent *E.coli* AG1 cells (Stratagene)
according to the supplier's instructions.

20 GVWP hydrophilic membranes (cat# GVWP14250,
Millipore) are placed on CAM 20 LB agar plates (Sambrook
et al., 1989). One membrane is added to each plate. Four
hundred microliters of the *E.coli* AG1 transformation
suspension from above are evenly spread over the surface

25 of each membrane. The plates are incubated for 16 hours
at 37 °C ambient temperatures.

Preparation of TAG-72-coated membranes:

A 1% dilution of partially purified tumor

30 associated glycoprotein-72 (TAG-72) produced in LS174 T-
cells is prepared in TBS (cat# 28376, Pierce). Ten
milliliters of the TAG dilution are placed in a petri
plate (cat# 8-757-14. Fisher) for future use.
Immobilon-P PVDF transfer membranes (cat# SE151103,
Millipore) are immersed in methanol. The membranes are

than rinsed three times in sterile double distilled
water. After the final wash, the excess water is
allowed to drain. Each of the membranes are placed in
10 milliliters of dilute TAG-72. The membranes are
incubated at ambient temperature from 1 hour with gentle
5   shaking. After incubation, the membranes are blocked
with Western blocking solution (25 mM Tris, 0.15 M NaCl,
pH 7.6; 1% BSA) for about 1 hour at ambient temperature.

10   Blocking solution is drained from the TAG
membranes. With the side exposed to TAG-72 facing up,
the membranes are placed onto fresh CAM 20 plates.
Resulting air pockets are removed. The bacterial
membranes are then added, colony side up, to a TAG
15   membrane. The agar plates are incubated for 24 to 96
hours at ambient temperatures.

      The orientation of the TAG-72 and bacterial
membranes are marked with permanent ink. Both membranes
20   are removed from the agar surface. The TAG-72 membrane
is placed in 20 ml of Western antibody buffer (TBS in
0.05% Tween-20, cat# P-1379, Sigma Chemical Co.; 1% BSA,
cat#3203, Biocell Laboratories) containing 0.2 ng of
CC49-Biotin probe antibody. The bacterial membranes are
25   replaced on the agar surface in their original
orientation and set aside. CC49-Biotin is allowed to
bind to the TAG membranes for 1 hour at 31 °C with
gentle shaking. The membranes are then washed three
times with TTBS (TBS, 0.05% Tween-20) for 5 minutes on
30   an orbital shaker at 300 rpm. Streptavidin alkaline
phosphatase (cat# 7100-04, Southern Biotechnology
Associates) is added to Western antibody buffer to
produce a 0.1% solution. The TAG-72 membranes are each
immersed in 16 milliliters of the streptavidin solution
and allowed to incubate for 30 minutes at 31 °C with

gentle shaking. After incubation, the membranes are washed as previously described. A final wash is then performed using Western alkaline phosphate buffer (8.4 g NaCO$_3$, 0.203 g MgCl$_2$-H$_2$O, pH 9.8), for 2 minutes at 200 rpm at ambient temperature. To develop the membranes,

5   Western blue stabilized substrate (cat# S384B, Promega) is added to each membrane surface. After 30 minutes at ambient temperatures, development of the membranes is stopped by rinsing the membranes three times with ddH$_2$O.

10 The membranes are then photographed. The membranes are then photograhed and clear zones are corelated with colonies on the hydrophilic membrane, set aside earlier. Colony(ies) are isolated for growth in culture and used to prepare plasmid DNA for sequencing and protein

15 preparation to evaluate specificity and affinity.

Identification of Hum4 V$_L$, human V$_H$ combinations using pATDFLAG.

20 In a second assay system, Hum4 V$_L$ - human V$_H$ combinations are discovered that bind to TAG-72 according to the schematic, *supra*, except for the following: at the assay step, IBI MII antibody is used as a probe to detect any Hum4 V$_L$ - V$_H$ SCFV combinations

25 that have bound to the hydrophobic membrane coated with TAG-72.

The plasmid pATDFLAG was generated from pSCFVUHH (see Figure 29) to incorporate a flag-coating sequence 3' of any human V$_H$ genes to be expressed

30 continguously with Hum4 V$_L$. The plasmid pATDFLAG, when digested with *Xho* I and *Nhe* I and purified becomes the human C$_H$ discovery plasmid containing Hum4 V$_L$ in this SCFV format. The plasmid pATDFLAG was generated as follows. Plasmid pSCFVUHH treated with *Xho* I and *Nhe* I (isolated and described above) was used in a ligation

reaction with the annealed FLAG and FLAGNC oligonucleotides.

FLAGC:
5'-TCGAGACAATGTCGCTAGCGACTACAAGGACGATGATGACAAATAAAAAC-3'

FLAGNC:
5'-CTAGGTTTTTATTTGTCATCATCGTCCTTGTAGTCGCTAGCGACATTGTC-3'

Equimolar amounts (1 x 10^{-10} moles of each of the oligonucleotides FLAGC and FLAGNC were mixed together using a ligation buffer (Stratagene). The sample is heated to 94 °C and is allowed to cool to below 35 °C before use in the ligation reaction below.

Ligation Reaction to Obtain pATDFLAG

COMPONENT	AMOUNT
pSCFVUHH *Xho* I/*Nhe* I vector	1.5 µl
ANNEALED FLAGC/FLAGNC	0.85 µl
10X Ligation buffer	2 µl
T4 DNA LIGASE	1 µl
10 MM ATP	2 µl
ddH₂O	12.65 µl

The reaction is carried out using the following components and amounts according the ligation protocol disclosed above. *E coli* AG1 cells (Stratagene) are transformed with 3 µl of the above ligation reaction and colonies selected using CAM 20 plates. Clones having

appropriate *Nhe* I, *Xho* I and *Nhe* I/*Xho* I restriction
patterns are selected for DNA sequencing.

The oligonucleotide used to verify the sequence
of the FLAG linker in PATDFLAG (see Figure 28) is called
5 PENPTSEQ: 5'-CTTTATGTAAGATGATGTTTTG-3. This
oligonucleotide is derived from the non-coding strand of
the *penP* terminator region. DNA sequencing is performed
using Sequenase™ sequencing kit (U.S. Biochemical,
Cleveland, OH) following the manufacturer's directions.
10 The DNA and deduced amino acid sequences of the Hum4 V$_L$
- UNIHOPE linker - FLAG peptide is shown in Figure 28.

Generating pSC49FLAG

15 The CC49V$_H$ is inserted into the sites of *Xho* I
- *Nhe* I pATDFLAG (see Figure 29) and evaluated for
biological activity with the purpose of serving as a
positive control for the FLAG assay system to detect
binding to TAG-72. The new plasmid, called pSC49FLAG
20 (see Figure 29) is generated as follows. The plasmid
pATDFLAG (5 mg, purified from a 2.5 ml culture by the
Magic Miniprep™ system (Promega) is treated with *Xho* I
and *Nhe* I and the large vector fragment purified as
described above for pSCFVUHH. The CC49 V$_H$ insert DNA
25 fragment is obtained by PCR amplification from pSCFVUHH
and oligonucleotides UNI3 as the 5' end oligonucleotide
and SC49FLAG as the 3' end oligonucleotide. The
resulting DNA and amino acid sequences of this SCFV
30 antibody, with the FLAG peptide at the C-terminus, is
shown in Figure 30. The PCR reaction is carried out
using 100 pmol each of the oligonucleotides, 0.1 ng of
pSCFVUHH target DNA (uncut) and the standard protocol
and reagents provided by Perkin Elmer Cetus. The DNA is
first gel purfied, then treated with *Xho* I and *Nhe* I to

generate sticky ends and purified from a 4%
polyacrylamide gel and electroeluted as described
earlier. The DNA vector (pATDFLAG treated with *Xho* I
and *Nhe* I) and the insert (CC49 V_H PCR product from
5 pSCFVUHH treated with *Xho* I and *Nhe* I) are ligated in a
1:1 molar ratio, using 100 ng vector DNA (Stratagene
kit) and used to transform *E.coli* AG1 competent cells
(Stratagene) according to the manufacturer's directions.
A colony with the correct plasmid DNA is picked as the
10 pSC49FLAG clone.

Ligation of pATDFLAG Vector with PCR Amplified Hum4 V_H
Inserts

       The protocol for the ligation reaction is as
15 follows:

COMPONENT	AMOUNT
DNA vector:pATDFLAG *Xho* I/*Nhe* I	2.5 µL
Hum V_H (X) DNA inserts: *Xho* I/*Nhe* I	6 µL
10 mM ATP (Stratagene)	2 µL
10X buffer (Stratagene)	2 µL
T4 DNA ligase (Stratagene)	1 µL
ddH$_2$O	6.5 µL

30   DNA vector, ATP, 10X buffer and ddH2O are
combined. DNA insert and T4 DNA ligase are then added.
Ligation reactions are then placed in a 4 L beaker
containing H2O at 18 °C. The temperature of the water

is gradually reduced by refrigeration at 4 °C overnight.
This ligation reaction generates pHum4 V_L – hum V_H (X).

Transformation of _E.coli_ AG1 with pHum4 V_L-Hum V_H (X) Ligation Mix

5   Transformation of pATDFLAG into competent _E.coli_
AG1 cells (Stratagene, La Jolla) is achieved following
the supplier's protocol.

IBI MII Anti-FLAG Antibody Plate Assay

10

          The first three steps, preparation of TAG-
coated membranes, plating of bacterial membranes, and
assembly of TAG and bacterial membranes, are the same as
those described in the CC49-Biotin Competition Plate
15   Assay.

          After the 24 hour incubation at ambient
temperatures, the membranes are washed with TTBS three
times at 250 rpm for four minutes. The MII antibody
20   (cat# IB13010, International Biotechnologies, Inc.) is
then diluted with TBS to a concentration ranging from
10.85 μg/ml to 0.03 μg/ml. Ten millilters of the
diluted antibody are added to each membrane. The
25   membranes are then incubated for 1 hour at ambient
temperatures and shaken on a rotary shaker at 70 rpm.
After incubation, the MII antibody is removed and the
membranes are washed three times at 250 rpm and ambient
temperatures for 5 minutes. The final wash is removed
30   and 20 milliters of a 1:2000 dilution of sheep anti-
mouse horseradish peroxidase linked whole antibody
(cat# NA931, Amersham) is prepared with TBS and added to
each membrane. The membranes are again incubated for 1
hour at ambient temperatures and 70 rpm. Following
incubation, the membranes are washed three times at 250

rpm and ambient temperature for 5 minutes each. Enzygraphic Webs (cat# IB8217051, International Biotechnologies, Inc.) are used according to develop the membranes, according to the manufacturer's instructions. The membranes are then photographed.

Instead of seeing a clear zone on the developed membrane for a positive Hum4 V_L-V_H (X) clone producing an SCFV that binds to TAG-72, (as seen with the competition screening assay) in this direct FLAG – detecting assay, a blue-purple spot is indicative of a colony producing a SCFV that has bound to the TAG-72 coated membrane. The advantage of using the FLAG system is that any Hum4 V_L – V_H SCFV combination that has bound to TAG-72 will be detected. Affinities can be measured by Scatchard analysis (Scatchard (1949), *supra*) and specificity by immunohistochemistry. These canidates could then be checked for binding to a specific epitope by using the competition assay, *supra*, and a competing antibody or mimetic, if desired.

The present invention is not to be limited in scope by the cell lines deposited since the deposited embodiment is intended astwo illustration of one aspect of the invention and all cell lines which are functionally equivalent are within the scope of the invention. Indeed, while this invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications could be made therein without departing from the spirit and scope of the appended claims.

Claims

1. A composite Hum4 V_L, V_H antibody having binding affinity for TAG-72 comprising

5

A. a light chain having a variable region (V_L), said V_L being encoded by a DNA sequence encoding at least a portion of a light chain variable region effectively homologous to the human Subgroup IV germline gene (Hum4 V_L); and

10

B. a heavy chain having a variable region (V_H), said V_H being encoded by a DNA sequence segment encoding at least a portion of a heavy chain variable region (V_H) which is capable of combining with the V_L to form a three dimensional structure having the ability to bind TAG-72.

15

2. The composite Hum4 VL, VH antibody of Claim 1, wherein the VL is further encoded by a human J gene segment.

20

3. The composite Hum4 V_L, V_H antibody according to Claim 1, wherein the V_H is encoded by a DNA sequence comprising a subsegment effectively homologous to the $V_H\alpha$TAG germline gene ($V_H\alpha$TAG).

25

4. The composite Hum4 V_L, V_H antibody of Claim 1, wherein the V_H is further encoded by an animal D gene segment and an animal J gene segment.

5. The composite Hum4 V_L, V_H antibody of Claim 1, wherein the variable region is derived from the variable regions of CC46, CC49, CC83 or CC92.

6. The composite Hum4 V_L, V_H antibody of Claim 1, wherein the V_H comprises (1) complementarity diversity regions (CDR) being encoded by a gene derived from the $V_H\alpha TAG$. and (2) framework segments, adjacent to the CDR segments. encoded by a human genes.

7. The composite Hum4 V_L, V_H antibody of Claim 1, wherein the light chain further comprises at least a portion of a human light chain (C_L) and the heavy chain further comprises at least a portion of a animal constant region (C_H).

8. The composite Hum4 V_L, V_H antibody of Claim 6, wherein the C_H is IgG1-4, IgM, IgA1, IgA2, IgD or IgE.

9. The composite Hum4 V_L, V_H antibody of Claim 7, wherein C_L is kappa or lambda.

10. A composite Hum4 V_L, V_H single chain antibody or immunoreactive fragment thereof comprising (a) a light chain having a variable region (V_L), said V_L being encoded by a DNA sequence encoding at least a portion of a light chain variable region (V_L) effectively homologous to the human Subgroup IV germline gene (Hum4 V_L); (b) a heavy chain having a variable region (V_H), said V_H being encoded by a DNA sequence segment encoding at least a portion of a heavy chain

variable region (V_H) and (c) a linker linking the V_H and V_L, wherein the polypeptide linker properly folds the V_H and V_L into a single chain antibody which is capable of forming a three dimensional structure having the ability to bind TAG-

5 72.

11. A composite Hum4 V_L, V_H antibody conjugate comprising the composite Hum4 V_L, V_H antibody of Claims 1 through 10 conjugated to an imaging marker or a therapeutic agent.

10 12. The composite Hum4 V_L, V_H antibody conjugate of Claim 11, wherein the imaging marker is selected from the group consisting of ^{125}I, ^{131}I, ^{123}I, ^{111}In, ^{105}Rh, ^{153}Sm, ^{67}Cu, ^{67}Ga, ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re, and ^{99m}Tc.

13. The composite Hum4 V_L, V_H antibody conjugate of

15 Claim 11, wherein the therapeutic agent is a drug or biological response modifier, radionuclide, or toxin.

14. The composite Hum4 V_L, V_H antibody conjugate of Claim 13, wherein the drug is methotrexate, adriamycin or interferon.

20 15. The composite Hum4 V_L, V_H antibody conjugate of Claim 13, wherein the radionuclide is ^{131}I, ^{90}Y, ^{105}Rh, ^{47}Sc, ^{67}Cu, ^{212}Bi, ^{211}At, ^{67}Ga, ^{125}I, ^{186}Re, ^{188}Re, ^{177}Lu, ^{99m}Tc, ^{153}Sm, ^{123}I or ^{111}In.

16. A composition comprising the composite Hum4 V_L, V_H

25 antibody of Claim 1 in a pharmaceutically acceptable, non-toxic, sterile carrier.

17. A composition comprising the composite Hum4 V_L, V_H antibodyof Claim 12 in a pharmaceutically acceptable, non-toxic, sterile carrier.

18. A composition comprising the composite Hum4 V_L, V_H antibody of Claim 13 in a pharmaceutically acceptable, non-toxic, sterile carrier.

19. A method for *in vivo* diagnosis of cancer which comprises administering to an animal a pharmaceutically effective amount of the composition of Claim 16 for the *in situ* detection of carcinoma lesions.

20. The method of Claim 19, wherein the animal is a human.

21. A method for the *in vivo* treatment of cancer which comprises administering to an animal a pharmaceutically effective amount of the composition of Claim 18.

22. The method of Claim 20, wherein the animal is a human.

23. A method for intraoperative therapy which comprises

(a) administering to an animal having at least one tumor a pharmaceutically effective amount of the composition of Claim 16, whereby the tumors are localized, and

(b) excising the tumors.

24. The method of Claim 23, wherein the animal is a human.

25. A cell capable of expressing the composite Hum4 V_L, V_H antibody or immunoreactive fragment thereof of Claim 1, said cell being transformed with

(A) a first DNA sequence encoding at least a
5   portion of a light chain variable region (V_L) effectively homologous to the human Subgroup IV germline gene (Hum4 V_L); and

(B) a second DNA sequence encoding at least a
10 portion of a heavy chain variable region (V_H) which is capable of combining with the V_L to form a three dimensional structure having the ability to bind TAG-72..

15   26. The cell of Claim 25 wherein the first and second DNA sequences are contained within at least one biologically functional expression vector.

27. A process for producing a composite Hum4
20 V_L, V_H antibody comprising at least the variable domains of the antibody heavy and light chains, in a single host cell, comprising the steps of:

A. transforming at least one host cell with
25

i) a first DNA sequence encoding at least a portion of a light chain variable region (V_L) effectively homologous to the human Subgroup IV germline gene (Hum4 V_L), and

30
ii) a second DNA sequence encoding at least a portion of a heavy chain variable region (V_H) which is capable of combining with the V_L to form a three dimensional structure having the ability to bind TAG-72, and

B.   independently expressing said first DNA sequence and said second DNA sequence in said transformed single host cell.

28.   The process according to Claim 27 wherein said first and second DNA sequences are present in at least one vector.

29.   The process according to Claim 28 wherein the antibody heavy and light chains of the composite Hum4 V_L, V_H antibody are expressed in the host cell are secreted therefrom as an immunologically functional antibody molecule or antibody fragment.

30.   The process of Claim 27, wherein the second DNA sequence encodes the V_H of CC46, CC49, CC83 or CC92.

31.   A process for preparing an antibody or antibody fragment conjugate which comprises contacting:
        the composite Hum4 V_L, V_H antibody of Claim 1 with an imaging marker or therapeutic agent.

32.   The process of Claim 31, wherein the imaging marker is ^{125}I, ^{131}I, ^{123}I, ^{111}In, ^{105}Rh, ^{153}Sm, ^{67}Cu, ^{67}Ga, ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re or ^{99m}Tc.

33.   The process of Claim 32, wherein the therapeutic agent is a radionuclide, drug or biological response modifier, toxin or another antibody.

Fig. 1

FIG.2

```
VH αTAG.                                           CCT
CC49                                               : : :
CC83                                               : : :

VH αTAG  TCTCTTCCTC CACCACCAAA TCCACCATTT GTAAATCAAC
CC49     .......... .......... .......... ..........
CC83     .......... .......... .......... ..........

VH αTAG  ATGTTAACAT ATCACAGAGT GGAGCAACAG AATCAGGGCA
CC49     .......... .......... .......... ..........
CC83     .......... .......... .......... ..........

VH αTAG  AAAATATGCT GAGAGATTTA TCCCTGTCGT TACAACCAAA
CC49     .......... ........T. .......... ..........
CC83     .......... .......... .......... ..........

VH αTAG  GCATCTGTCT AGAATTCATA AAAACTTTAT GGGATACATT
CC49     .......... .......... .......... ..........
CC83     .......... .......... .......... ..........

VH αTAG  TCCTCAGAGA GGAATAGGAT TTGGACCTGA CGATCCTGCT
CC49     .......... .......... .......... ..........
CC83     .......... .......... .......... ..........
```
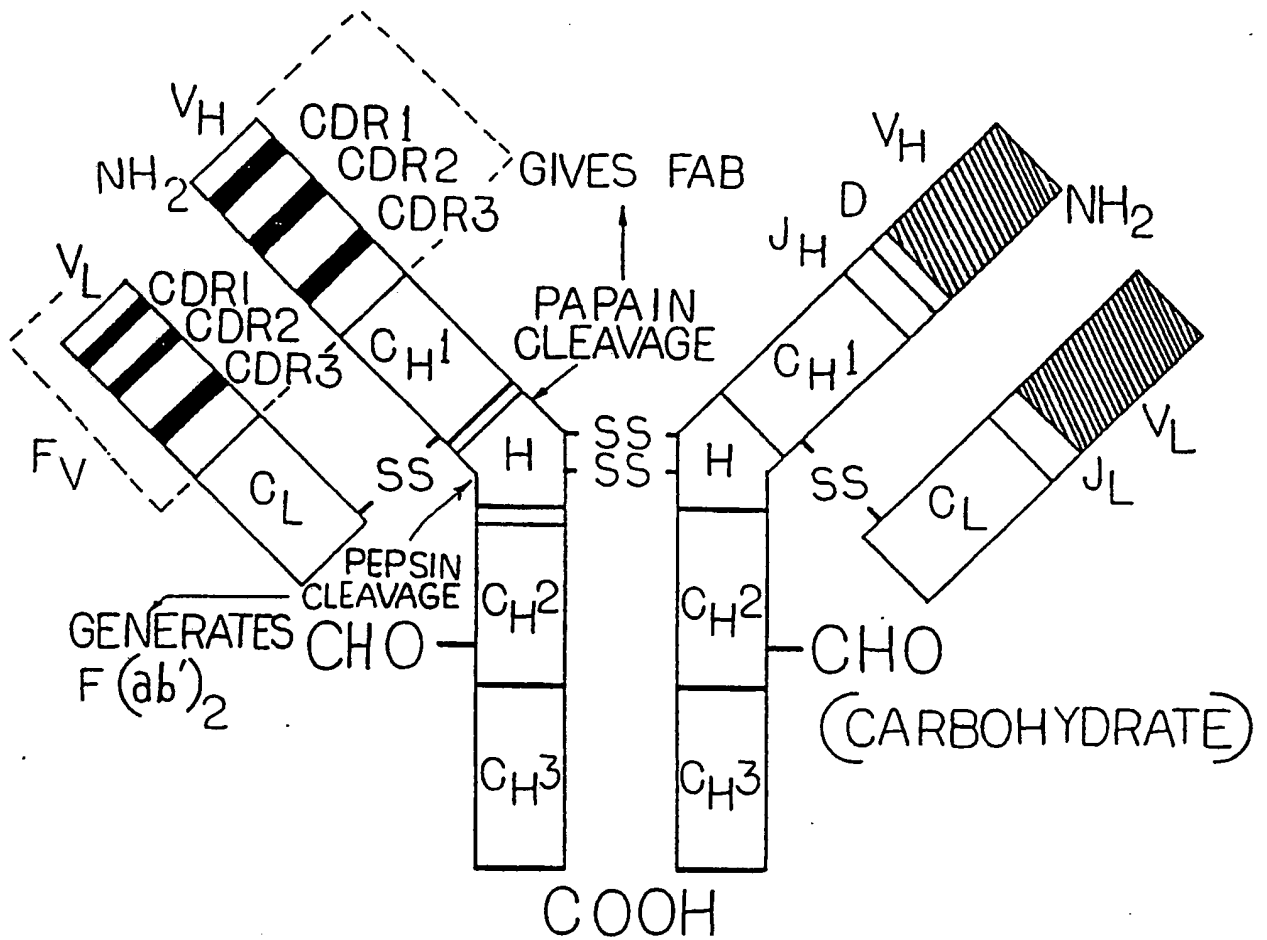
FIG. 2 (CONT.)

```
VH αTAG    GCCCGAGCCA  TGTGATGACA  GTTCTTCTCC  AGTTGAACTA
CC49       ..........  ..........  ..........  ..........
CC83       ..........  ..........  ..........  ..........

VH αTAG    GGTCCTTATC  TAAGAAATGC  ACTGCTCATG  AATATGCAAA
CC49       ..........  ..........  ..........  ..........
CC83       ..........  ..........  ..........  ..........

VH αTAG    TCACCCGAGT  CTATGGCAGT  AAATACAGAG  ATGTTCATAC
CC49       ..........  ..........  ..........  ..........
CC83       ..........  ..........  ..........  ..........

VH αTAG    CATAAAAACA  ATATATGATC  AGTGTCTTCT  CCGCTATCCC
CC46       ..........  ..........  ..........  ..........
CC49       ..........  ...G......  ..........  ..........
CC83       ..........  ..........  ..........  ..........
CC92       ..........  ..........  ..........  ..........
```

FIG. 2 (CONT.)

$V_H\alpha$TAG	TGGACACACT	GACTCTAACC	ATG	GAA	TGG	AGC	TGG
CC46	....	....	:	:	:	:	:
CC49	....	....	:	:	:	:	:
CC83	....	....	:	:	:	:	:
CC92	....	....	:	:	:	:	:

$V_H\alpha$TAG	GTC	TTT	CTC	TTC	CTG	TCA	GTA	ACT	ACA	G
CC46	:	:	:	:	:	:	:	:	:	:
CC49	:	:	:	:	:	:	:	:	:	:
CC83	:	:	:	:	:	:	:	:	:	:
CC92	:	:	:	:	:	:	:	:	:	:

$V_H\alpha$TAG	GTAAGGGGCT	CACCATTTCC	AAATCTAAAG	TGGAGTCAGG
CC46	....	....	....	....
CC49	....	....	....	....
CC83	....	....	....	....
CC92	....	....	....	....

FIG. 2 (CONT.)

```
VHαTAG   GCCTGAGGTG  ACAAAGATAT  CACCTTTGGC  TTTCCACAG
CC46     . . . . .   . . . . . . . . . .     . . . . . . . . .
CC49     . . . . .   . . . . .G. . . . .G    . . . . . . . . .
CC83     . . . . .   . . . . . . . . . .     . . . . . . . . .
CC92     . . . . .   . . . . . . . . . .     . . . . . . . . .


VHαTAG   GT GTC CAC  TCC CAG GTT  CAG CTG CAG  CAG TCT
CC46     . . . . . . . .  .A T. . . . .   . .A . . .  . . .
CC49     . . . . . . . .  . . . . . . .   T. . . .  . . .
CC83     . . . . . . . .  . . . . . . .   T. . . .  . . .
CC92     . . . . . . . .  . . . .A . . .   . . . .  . . .


VHαTAG   GAC GCT GAG  TTG GTG AAA  CCT GGG GCT  TCA GTG
CC46     . . . . . . . .  . . . . .G . . .   . . . .  . . .
CC49     . . . . . . . .  . . . . . . . .   . . . .  . . .
CC83     . . . . . . . .  . . . . . . . .   . . . .  . . .
CC92     . . . . . . . .  . . . . . . . .   . . . .  . . .
```

FIG. 2 (CONT.)

```
VHαTAG  AAG ATA TCC TGC AAG GCT TCT GGC TAC ACC TTC
CC46    ... .T. ... ... ... ... ... ... ... ... ...
CC49    ... .T. ... ... ... ... ... ... ... ... ...
CC83    ... .T. ... ... ... ... ... ... ... ... ...
CC92    ... ... ... ... ... ... ... ... ... ... ...

                        CDR1
VHαTAG  ACT GAC CAT GCT ATT CAC TGG GTG AAG CAG AAG
CC46    ... ... ... ... ... ... ... ... ... ... ...
CC49    ... ... .A. ... ... ... ... .A. ... ... ..C
CC83    ... ... ... ... ... ... ... ... ... ... ...
CC92    ... ... ... ... ... ... ... ... ... ... .A

                                        TAT ATT
VHαTAG  CCT GAA CAG GGC CTG GAA TGG ATT GGA TAT ATT
CC46    ... ... ... ... ... ... ... ... ... T.. ...
CC49    ... ... ... ... ... ... ... ... ... T.. ...
CC83    ... ... ... ... ... ... ... ... ... ... ...
CC92    ... ... ... ... ... ... ... ... ... ... ...

                        CDR2
VHαTAG  TCT CCC GGA AAT GGT GAT ATT AAG TAC AAT GAG
CC46    ... ... ... ... ... ... ... ... ... ... ...
CC49    ... ... ... A. T.. .A. ... ... ... ... ...
CC83    ... ... ... A. ... ... ... ..A ... ... ...
CC92    ... ... ... A. ... ... ... ... ... ... ...
```

FIG. 2 (CONT.)

CDR2

```
VHαTAG   AAG TTC AAG GGC  AAG GCC ACA CTG ACT GCA GAC
CC46     .G. ... ... ...  ... ... ... ... ... ... ...
CC49     ... ... ... ...  ... ... ... ... ... ... ...
CC83     ... ... ... ...  ... ... ... ... ... ... ...
CC92     ... ... ... ..T  ... ... ... ... ... ... ...

VHαTAG   AAA TCC TCC AGC ACT GCC TAC ATG CAG CTC AAC
CC46     ... ... ... ... ... ... ... ... ... ... ...
CC49     ... .C.. ... ... ... ... ... G.. T.. ... ...
CC83     ... ... ... ..T ... ... ... ... ... .A. ...
CC92     ... ... ... .A. ... .T. ... ... ... ... ...

VHαTAG   AGC CTG ACA TCT GAG GAT TCT GCA GTG TAT TTC
CC46     ... ... ... ... ... ... ... ... ... ... ...
CC49     ... ... ... ... .G. ..C ... ... ... ... ...
CC83     ... ..C ... ... ... ... ... ... ... ... ...
CC92     ... ... ... ... ... ... ... ... ... ... ...
```

FIG. 2 (CONT.)

```
V_H αTAG   TGT AAA AGA  CACAGTGTTG TAACCACATC CTGAGTGTGT
CC46       ... ... .CG G.C GGC TAC GGG GTT GCT TTC TGG GGC
CC49       ... ... .C. ... TCC CTG AAT ATG GCC TAC TGG GGT
CC83       ... ... .G. ... TCC TTC TAC GGC --- TGG GGC
CC92       ... ... .C. ... TCT CTA TCC GGG GAC TCC TGG GGC
                                       ├──── CDR3 ────┤

V_H αTAG   CAGAAATCCT GGGGGAGCAG AAAGATACAC TGGGACTGAG
CC46       CAA GGG ACT CTG GTC ACT GTC TCT GCA G
CC49       CAA GGA ACC TCA GTC ACC GTC TCA G
CC83       CAA GGC ACC ACC CTC ACA GTC TCA G
CC92       CAG GGC ACC ACC ACT CTC ACA GTC TCC TCA G

V_H αTAG   AAGACAGAAA AATTAATCCT TAGACTTGCT CAGAAATCGT

V_H αTAG   AATTTTGAAT GCCTATTTAT TTCATCTTGC TCACACACCT

V_H        ATATTGCTTT TGTAAGCTT
```

FIG. 2 (CONT.)

```
                                    ┌── CDR2 ──────►
          AAG TTC AAG GGC│AAG GCC ACA CTG ACT GCA GAC
VH∝TAG
CC46      ... ... ... ...  ... ... ... ... ... ... ...
CC49      .G. ... ... ...  ... ... ... ... ... ... ...
CC83      ... ... ... ...  ... ... ... ... ... ... ...
CC92      ... ... ... ..T  ... ... ... ... ... ... ...

          AAA TCC TCC AGC ACT GCC TAC ATG CAG CTC AAC
VH∝TAG
CC46      ... ... ... ... ... ... ... ... ... T.. ...
CC49      ... ... ... ... ... ... ... G.. ... ... ...
CC83      ... ... ... ..T ... ... ... ... ..A ... ...
CC92      ... C.. ... A.. .T. ... ... ... ... ... ...

          AGC CTG ACA TCT GAG GAT TCT GCA GTG TAT TTC
VH∝TAG
CC46      ... ... ... ... ... ..C ... ... ... ... ...
CC49      ... ... ... ... ... ... ... ... ... ... ...
CC83      ... ... ..C ... ... ... ... ... ... ... ...
CC92      ... ... ... ... ... ... ... ... ... ... ...
```

FIG. 2 (CONT.)

V$_H$αTAG	TGT	AAA	AGA	CACAGTGTTG	TAACCACATC	CTGAGTGTGT			
CC46	...	...	.CG	G.C	GGC	TAC	GGG	TTT	GCT TTC TGG GGC
CC49	...	...	.C.		TCC	CTG	AAT	ATG	GCC TAC TGG GGT
CC83	...	...	.G.		TCC	TTC	TAC	GGC	--- TGG GGC
CC92	...	...	.C.		TCT	CTA	TCC	GGG	AAC TCC TGG GGC

CDR3

V$_H$αTAG	CAGAAATCCT	GGGGGAGCAG	AAAGATACAC	TGGGACTGAG	
CC46	CAA	GGG	ACT	CTG GTC ACT GTC TCT GCA	G
CC49	CAA	GGA	ACC	TCA GTC ACC GTC TCC TCA	G
CC83	CAA	GGC	ACC	ACC CTC ACA GTC TCC TCA	G
CC92	CAG	GGC	ACC	ACC ACT CTC ACA GTC TCC TCA	G

V$_H$αTAG   AAGACAGAAA   AATTAATCCT   TAGACTTGCT   CAGAAATCGT

V$_H$αTAG   AATTTTGAAT   GCCTATTTAT   TTCATCTTGC   TCACACACCT

V$_H$αTAG   ATATTGCTTT   TGTAAGCTT

FIG.3


```
                    ─── LEADER PEPTIDE ───
            ┌─19                           -10
          ┌→│ Met Glu Trp Ser Trp Val Phe Leu Phe Phe
VHαTAG    │
CC46      · · · · · · · · · · ·
CC49      · · · · · · · · · · ·
CC83      · · · · · · · · · · ·
CC92      · · · · · · · · · · ·

          ↑
          │ Leu Ser Val Thr Thr Gly Val His Ser Gln│
VHαTAG    │
CC46      · · · · · · · · · · ·
CC49      · · · · · · · · · · ·
CC83      · · · · · · · · · · ·
CC92      · · · · · · · · · · ·

                                            10
            Val Gln Leu Gln Gln Ser Asp Ala Glu Leu
VHαTAG      Phe
CC46        · · · · · · · · · · ·
CC49        · · · · · · · · · · ·
CC83        · · · · · · · · · · ·
CC92        · · · · · · · · · · ·
```

FIG. 3 (CONT.)

```
                                                    20
VHαTAG   Val Lys Pro Gly Ala Ser Val Lys Ile Ser
CC46     Arg  .   .   .   .   .   .   .   .   .
CC49      .   .   .   .   .   .   .   .   .   .
CC83      .   .   .   .   .   .   .   .   .   .
CC92      .   .   .   .   .   .   .   .   .   .

                                        30        ↓
                                            CDR1 →
VHαTAG   Cys Lys Ala Ser Gly Tyr Thr Phe Thr | Asp
CC46      .   .   .   .   .   .   .   .   .   .
CC49      .   .   .   .   .   .   .   .   .   .
CC83      .   .   .   .   .   .   .   .   .   .
CC92      .   .   .   .   .   .   .   .   .   .

                                                    40
              CDR1
         His Ala Ile His | Trp Val Lys Gln Lys Pro
VHαTAG
CC46      .   .   .   .   .   .   .   .   .   .
CC49      .   .   .   .   .   .   .   .   .   .
CC83      .   .   .   .   .  Asn  .   .   .   .
CC92      .   .   .   .   .   .   .   .   .   .

                                           50      ↓
VHαTAG   Glu Gln Gly Leu Glu Trp Ile Gly | Tyr Ile
CC46      .   .   .   .   .   .   .   .   .  Phe
CC49      .   .   .   .   .   .   .   .   .  Phe
CC83      .   .   .   .   .   .   .   .   .   .
CC92      .   .   .   .   .   .   .   .   .   .
```

FIG. 3 (CONT.)

CDR2

                           60

V$_H$αTAG	Ser	Pro	Gly	Asn	Gly	Asp	Ile	Lys	Tyr	Asn
CC46	.	.	.	.	.	.	.	.	.	.
CC49	.	.	.	Asp	.	Phe	.	.	.	.
CC83	.	.	.	Asp	.	.	.	.	.	.
CC92	.	.	.	Asp	.	.	.	.	.	.

                           70

V$_H$αTAG	Glu	Lys	Phe	Lys	Gly	Lys	Ala	Thr	Leu	Thr
CC46	.	.	.	.	.	.	.	.	.	.
CC49	.	Arg	.	.	.	.	.	.	.	.
CC83	.	.	.	.	.	.	.	.	.	.
CC92	.	.	.	.	.	.	.	.	.	.

                           80

V$_H$αTAG	Ala	Asp	Lys	Ser	Ser	Ser	Thr	Ala	Tyr	Met
CC46	.	.	.	.	.	.	.	.	.	.
CC49	.	.	.	.	.	.	.	.	.	Val
CC83	.	.	.	Pro	.	.	.	.	.	.
CC92	.	.	.	.	Pro	Asn	.	Val	.	.

FIG. 3 (CONT.)

				110			
CC46	Gly	Thr	Leu	Val	Thr	Val	Ser	Ala
CC49	Gly	Thr	Ser	Val	Thr	Val	Ser	Ser
CC83	Gly	Thr	Thr	Leu	Thr	Val	Ser	Ser
CC92	Gly	Thr	Thr	Leu	Thr	Val	Ser	Ser

FIG. 3 (CONT.)

VHαTAG	Gln	Leu	Asn	Ser	Leu	Thr	Ser	Glu	Asp	Ser
CC46	Phe	.	.	.	.	.	.	.	.	.
CC49	.	.	.	.	.	.	.	.	.	.
CC83	.	.	.	.	.	.	.	.	.	.
CC92	.	.	.	.	.	.	.	.	.	.

90

VHαTAG	Ala	Val	Tyr	Phe	Cys	Lys	Arg
CC46	.	.	.	.	.	Thr	Gly
CC49	.	.	.	.	.	Thr	.
CC83	.	.	.	.	.	Arg	.
CC92	.	.	.	.	.	Thr	.

CDR3 — 105

			CDR3					105	
CC46	Gly	Tyr	Gly	Val	Ala	Phe	Trp	Gly	Gln
CC49	Ser	Leu	Asn	Met	Ala	Tyr	Trp	Gly	Gln
CC83	Ser	Phe	Tyr	Gly	Asn	-	Trp	Gly	Gln
CC92	Ser	Leu	Ser	Gly	Asp	Ser	Trp	Gly	Gln

FIG. 3 (CONT.)

VHαTAG	Gln	Leu	Asn	Ser	Leu	Thr	Ser	Glu	Asp	Ser
CC46	.	Phe	.	.	.	.	.	.
CC49	.	.	.	.	.	.	.	.
CC83	.	.	.	.	.	.	.	.
CC92	.	.	.	.	.	.	.	.

90

VHαTAG	Ala	Val	Tyr	Phe	Cys	Lys	Arg
CC46	.	.	.	.	.	Thr	Gly
CC49	.	.	.	.	.	Thr	.
CC83	.	.	.	.	.	Arg	.
CC92	.	.	.	.	.	Thr	.

CDR3   **105**

CC46	Gly	Tyr	Gly	Phe	Ala	Phe	Trp	Gly	Gln
CC49	Ser	Leu	Asn	Met	Ala	Tyr	Trp	Gly	Gln
CC83	Ser	Phe	Tyr	Gly	Asn	-	Trp	Gly	Gln
CC92	Ser	Leu	Ser	Gly	Asn	Ser	Trp	Gly	Gln

FIG. 4

```
                      Met Glu Lys Leu Trp Phe
   1 5'-GAA TTC ATG GAA AAA CTT TGG TTC

   7      Leu Leu Leu Leu Leu Thr Ile Pro
  25      TTG CTT CTG CTG CTG ACC ATC CCT

  15      Ser Trp Val Leu Ser Gln Ile Thr
  49      TCA TGG GTC TTG TCC CAG ATC ACC

  23      Leu Lys Glu Ser Gly Pro Thr Leu
  73      TTG AAG GAG TCT GGT CCT ACN CTG

  31      Val Lys Pro Thr Gln Thr Leu Thr
  97      GTG AAA CCC ACA CAG ACC CTC ACG

  37      Leu Thr Cys Thr Phe Ser Gly Phe
 121      CTG ACC TGC ACC TTC TCT GGG TTC
                              ┌──────── CDR1 ────────
  47      Ser Leu Ser │Thr His Gly Val Gly
 145      TCA CTC AGC │ACT CAT GGA GTG GGT
       ┌─────────────┐
  55   │Val Gly│ Trp Ile Arg xxx xxx Pro
 169   │GTG GGC│ TGG ATC CGT NNN NNC CCA

  63      Gly Lys Ala Leu Glu Trp Leu Ala
 193      GGA AAG GCC CTG GAG TGG CTT GCA
        ┌──────────────── CDR2 ────────────
  71    │Leu Ile Tyr Trp Asp Asp Asp Lys
 217    │CTC ATT TAT TGG GAT GAT GAT AAG
        └──────────────────────────────────┐
  79      Arg Tyr Ser Pro Ser Leu Lys Ser │
 241      CGC TAC AGC CCA TCT CTG AAG AGC │
```

FIG. 4 (CONT.)

```
87     Arg Leu Thr Ile Thr Lys Asp Thr
265    AGG CTC ACC ATC ACC AAG GAC ACC

95     Ser Lys Asn Gln Val Ile Leu Thr
289    TCC AAA AAC CAG GTG ATC CTT ACA

103    Met Thr Asn Met Asp Pro Val Asp
313    ATG ACC AAC ATG GAC CCT GTG GAC

111    Thr Ala Thr Tyr Tyr Cys Ala His
337    ACA GCC ACA TAT TAT TGT GCA CAC
       ◄─────────────── CDR3 ───────────►
119    Gly Leu Pro Ser Met Val Lys Asn
361    GGG CTG CCA TCT ATG GTT AAG AAC

127    Trp Gly Gln Gly Thr Thr Val Thr
385    TGG GGC CAA GGG ACC ACG GTC ACC

135    Val Ser Ser Gly Ser
409    GTC TCC TCA GGG AGT-3'
```

FIG. 4

```
                  Met Glu Lys Leu Trp Phe
  1 5'-GAA TTC ATG GAA AAA CTT TGG TTC

  7       Leu Leu Leu Leu Leu Thr Ile Pro
 25       TTG CTT CTG CTG CTG ACC ATC CCT

 15       Ser Trp Val Leu Ser Gln Ile Thr
 49       TCA TGG GTC TTG TCC CAG ATC ACC

 23       Leu Lys Glu Ser Gly Pro Thr Leu
 73       TTG AAG GAG TCT GGT CCT ACG CTG

 31       Val Lys Pro Thr Gln Thr Leu Thr
 97       GTG AAA CCC ACA CAG ACC CTC ACG

 37       Leu Thr Cys Thr Phe Ser Gly Phe
121       CTG ACC TGC ACC TTC TCT GGG TTC
                            ┌──────── CDR1 ────────
 47       Ser Leu Ser │Thr His Gly Val Gly
145       TCA CTC AGC │ACT CAT GGA GTG GGT

          ┌─────────┐
 55       │Val Gly │Trp Ile Arg Gln Pro Pro
169       │GTG GGC │TGG ATC CGT CAG CCC CCA

 63       Gly Lys Ala Leu Glu Trp Leu Ala
193       GGA AAG GCC CTG GAG TGG CTT GCA
          ┌──────────────── CDR2 ────────────────
 71       │Leu Ile Tyr Trp Asp Asp Asp Lys
217       │CTC ATT TAT TGG GAT GAT GAT AAG

 79       Arg Tyr Ser Pro Ser Leu Lys Ser│
241       CGC TAC AGC CCA TCT CTG AAG AGC│
```

FIG. 5

MOUSE GERMLINE J-H GENES
FROM pNP9

```
5'-GGATCCTGGC  CAGCATTGCC  GCTAGGTCCC
   TCTCTTCTAT  GCTTTCTTTG  TCCCTCACTG
   GCCTCCATCT  GAGATAATCC  TGGAGCCCTA
   GCCAAGGATC  ATTTATTGTC  AGGGGTCTAA
   TCATTGTTGT  CACAATGTGC  CTGGTTTGCT
   TACTGGGGCC  AAGGGACTCT  GGTCACTGTC
   TCTGCAGGTG  AGTCCTAACT  TCTCCCATTC
   TAAATGCATG  TTGGGGGGAT  TCTGAGCCTT
   CAGGACCAAG  ATTCTCTGCA  AACGGGAATC
   AAGATTCAAC  CCCTTTGTCC  CAAAGTTGAG
   ACATGGGTCT  GGGTCAGGGA  CTCTCTGCCT
   GCTGGTCTGT  GGTGACATTA  GAACTGAAGT
   ATGATGAAGG  ATCTGCCAGA  ACTGAAGCTT
   GAAGTCTGAG  GCAGAATCTT  GTCCAGGGTC
   TATCGGACTC  TTGTGAGAAT  TAGGGGCTGA
   CAGTTGATGG  TGACAATTTC  AGGGTCAGTG
   ACTGTCAGGT  TTCTCTGAGG  TGAGGCTGGA
   ATATAGGTCA  CCTTGAAGAC  TAAAGAGGGG
   TCCAGGGGCT  TTTCTGCACA  GGCAGGGAAC
   AGAATGTGGA  ACAATGACTT  GAATGGTTGA
   TTCTTGTGTG  ACACCAAGAA  TTGGCATAAT
   GTCTGAGTTG  CCCAAGGGTG  ATCTTAGCTA
   AAAACCCACT  ATTGTGATTA  CTATGCTATG
   GACTACTGGG  GTCAAGGAAC  CTCAGTCACC
   GTCTCCTCAG  GTAAGAATGG  CCTCTCCAGG
   TCTTTATTTT  TAACCTTTGT  TATGGAGTTT
   TCTGAGCATT  GCAGACTAAT  CTTGGATATT
   TGCCCTGAGG  GAGCCGGCTG  AGAGAAGTTG
   GGAAATAAAT  CTGTCTAGGG  ATCTCAGAGC
   CTTTAGGACA  GATTATCTCC  ACATCTTTGA
   AAAACTAAGA  ATCTGTGTGA  TGGTGTTGGT
   GGAGTCCCTG  GATGATGGGA  TAGGGACTTT
```

FIG. 5 (CONT.)

```
GGAGGCTCAT  TTGAGGGAGA  TGCTAAAACA
ATCCTATGGC  TGGAGGGATA  GTTGGGGCTG
TAGTTGGAGA  TTTTCAGTTT  TTAGAATGAA
GTATTAGCTG  CAATACTTCA  AGGACCACCT
CTGTGACAAC  CATTTTATAC  AGTATCCAGG
CATAGGGACA  AAAAGTGGAG  TGGGGCACTT
TCTTTAGATT  TGTGAGGAAT  GTTCCACACT
AGATTGTTTA  AAACTTCATT  TGTTGGAAGG
AGCTGTCTTA  GTGATTGAGT  CAAGGGAGAA
AGGCATCTAG  CCTCGGTCTC  AAAAGGGTAG
TTGCTGTCTA  GAGAGGTCTG  GTGGAGCCTG
CAAAAGTCCA  GCTTTCAAAG  GAACACAGAA
GTATGTGTAT  GGAATATTAG  AAGATGTTGC
TTTTACTCTT  AAGTTGGTTC  CTAGGAAAAA
TAGTTAAATA  CTGTGACTTT  AAAATGTGAG
AGGGTTTTCA  AGTACTCATT  TTTTTAAATG
TCCAAAATTT  TTGTCAATCA  ATTTGAGGTC
TTGTTTGTGT  AGAACTGACA  TTACTTAAAG
TTTAACCGAG  GAATGGGAGT  GAGGCTCTCT
CATACCCTAT  TCAGAACTGA  CTTTTAACAA
TAATAAATTA  AGTTTAAAAT  ATTTTTAAAT
GAATTGAGCA  ATGTTGAGTT  GAGTCAAGAT
GGCCGATCAG  AACCGGAACA  CCTGCAGCAG
CTGGCAGGAA  GCAGGTCATG  TGGCAAGGCT
ATTTGGGAA   GGGAAAATAA  AACCACTAGG
TAAACTTGTA  GCTGTGGTTT  GAAGAAGTGG
TTTTGAAACA  CTCTGTCCAG  CCCCACCAAA
CCGAAAGTCC  AGGCTGAGCA  AAACACCACC
TGGGTAATTT  GCATTTCTAA  AATAAGTTGA
GGATTCAGCC  GAAACTGGAG  AGGTCCTCTT
TTAACTTATT  GAGTTCAACC  TTTTAATTTT
AGCTTGAGTA  GTTCTAGTTT  CCCCAAACTT
AAGTTTATCG  ACTTCTAAAA  TGTATTTAGA
ATTC-3'
```

Fig. 6
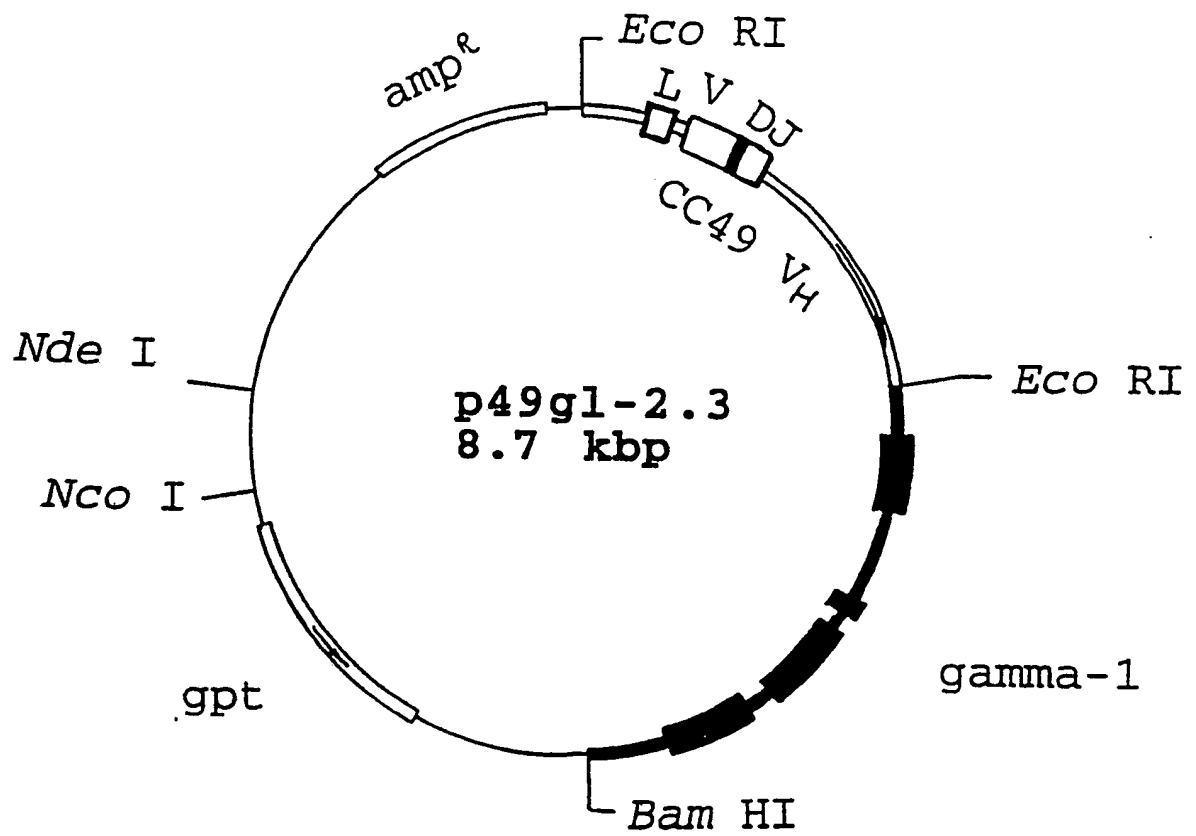
FIG. 7
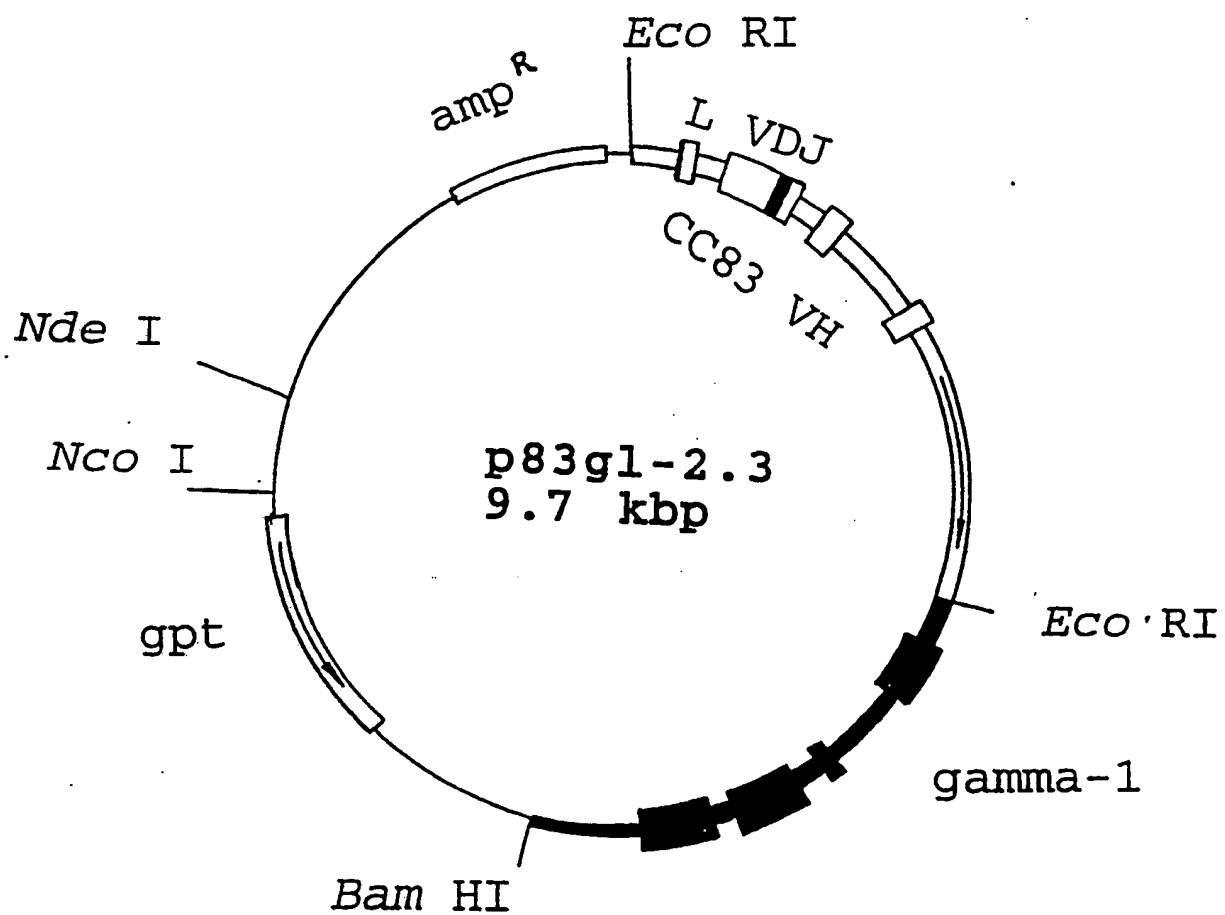
FIG. 8

HUMVL (+), 26-MER:
(*Cla* I)
5'- GAAGAGT**ATC GAT**AAAATTT ATTGAG-3'

HUMVL(-), 98-MER:

(SPLICE SITE)⌐
(*Hind* III) |
5'-CATT**AAGCTT** AGAAAAGTGT ACTTACGTTT
GATCACCACC TTGGTCCCTC CGCCGAAAGT
GAGAGGATAA CTATAATATT GCTGACAGTA
ATAAACTG-3'

FIG. 9

HJ4:

Leu Thr Phe Gly Gly Gly Thr Lys
CTC ACT TTC GGC GGA GGG ACC AAG

Val Glu Ile Lys A(rg)
GTG GAG ATC AAA C GTAAGTGCAC

TTTCCTAA

FIGURE 9: Human J4 (HJ4) amino acid and DNA sequences.The first two amino acids (Leu-Thr) complete the CDR3 region, the remainder make up the FR4 region. The (↓) indicates the splice site and the beginning of the intron between the J and C exons. DNA sequence underlined in HJ4 represents a part of the sequence used for the 3' end PCR oligo HUMVL(-).

FIG. 10

```
       Cla I
5' ATCGATAAAA TTTATTGAGA ATTGTTTTAT TATGATTAAC  3418
3' TAGCTATTTT AAATAACTCT TAAACAAATA ATACTAATTG

   AGAGGTAAAA GCCAGTATAT TACTGATTAA TATAGGTAAA  3458
   TCTCCATTTT CGGTCATATA ATGACTAATT ATATCCATTT
           *

   AGGCAGTTAA GAAATTGGGA ATGCTTTCTC TTCTGCTTTC  3498
   TCCGTCAATT CTTTAACCCT TACGAAAGAG AAGACGAAAG

   TTCTACGATG CACAAGGCGT TTCACATTTA TGCCCCTATG  3538
   AAGATGCTAC GTGTTCCGCA AAGTGTAAAT ACGGGGATAC

   AAAATTACTA GGCTGTCCTA GTCATTAGAT CTTTCAGCAG  3578
   TTTTAATGAT CCGACAGGAT CAGTAATCTA GAAAGTCGTC

   TTTGTAGTTT TAGAGCTTCT AAGTTGACTT CTGTCTTTTC  3618
   AAACATCAAA ATCTCGAAGA TTCAACTGAA GACAGAAAAG

   TATTCATACA ATTACACATT CTGTGATGAT ATTTTTGGCT  3658
   ATAAGTATGT TAATGTGTAA GACACTACTA TAAAAACCGA
                          HUMLIN1 (-)
```

FIG. 10 (CONT.)

```
CTTGATTTAC ATTGGGTACT TTCACAACCC ACTGCTCATG 3698
GAACTAAATG TAACCCATGA AAGTGTTGGG TGACGAGTAC

AAATTTGCTT TTGTACTACT GGTTGTTTTT GCATAGGCCC 3738
TTTAAACGAA AACATGATGA CCAACAAAAA CGTATCCGGG

CTCCAGGCCA CGACCAGGTG TTTGGATTTT ATAAACGGGC 3778
GAGGTCCGGT GCTGGTCCAC AAACCTAAAA TATTTGCCCG

CGTTTGCATT GTGAACTGAG CTACAACAGG CAGGCAGGGG 3818
GCAAACGTAA CACTTGACTC GATGTTGTCC GTCCGTCCCC

           Met Val Leu Gln Thr Gln Val Leu Ile -10
CAGCAAG ATG GTG TTG CAG ACC CAG GTC TTC ATT 3852
GTCGTTC TAC CAC AAC GTC TGG GTC CAG AAG TAA

Ser Leu Leu Leu Trp Ile Ser G Intron
TCT CTG TTG CTC TGG ATC TCT G GTGA GGAATTAAAAA -4
AGA GAC AAC GAG ACC TAG AGA C CACT CCTTAATTTT 3888

AGTGCCACAG TCTTTTCAGA GTAATATCTG TGTAGAAATA
TCACGGTGTC AGAAAGTCT CATTATAGAC ACATCTTTAT
            HUMLIN2(-)
```

FIG. 10 (CONT.)

```
AAAAAATTA  AGATATAGTT  GGAAATAATG  ACTATTTCCA
TTTTTTAAT  TCTATATCAA  CCTTTATTAC  TGATAAAGGT
            Bam HI
ATATGGATCC  AATTATCTGC  TGACTTATAA  TACTACTAGA
TATACCTAGG  TTAATAGACG  ACTGAATATT  ATGATGATCT

AAGCAAATTT  AAATGACATA  TTTCAATTAT  ATCTGAGACA
TTCGTTTAAA  TTTACTGTAT  AAAGTTAATA  TAGACTCTGT

GCGTGTATAA  GTTTATGTAT  AATCATTGTC  CATTACTGAC
CGCACATATT  CAAATACATA  TTAGTAACAG  GTAATGACTG

TACAG
ATGTC
```

```
                        +1
ly  Ala  Tyr  Gly  Asp  Ile  Val  Met  Thr  Gln  Ser
GT  GCC  TAC  GGG  GAC  ATC  GTG  ATG  ACC  CAG  TCT      7
CA  CGG  ATG  CCC  CTG  TAG  CAC  TAC  TGG  GTC  AGA   4125
```

FIG. 10 (CONT.)

```
                                       CDR1
Pro Asp Ser Leu Ala Val Ser Leu Gly Glu Arg Ala Thr 20
CCA GAC TCC CTG GCT GTG TCT CTG GGC GAG AGG GCC ACC 4164
GGT CTG AGG GAC CGA CAC AGA GAC CCG CTC TCC CGG TGG

Ile Asn Cys Lys Ser Ser Gln Ser Val Leu Tyr Ser Ser 27F
ATC AAC TGC AAG TCC AGC CAG AGT GTT TTA TAC AGC TCC 4203
TAG TTG ACG TTC AGG TCG GTC TCA CAA AAT ATG TCG AGG

Asn Asn Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro 40
AAC AAT AAG AAC TAC TTA GCT TGG TAC CAG CAG AAA CCA 4242
TTG TTA TTC TTG ATG AAT CGA ACC ATG GTC GTC TTT GGT

HUMLCDR1(-)                                    CDR2
Gly Gln Pro Pro Lys Leu Leu Ile Tyr Trp Ala Ser Thr 53
GGA CAG CCT CCT AAG CTG CTC ATT TAC TGG GCA TCT ACC 4281
CCT GTC GGA GGA TTC GAC GAG TAA ATG ACC CGT AGA TGG

Arg Glu Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly 66
CGG GAA TCC GGG GTC CCT GAC CGA TTC AGT CGC AGT CGC 4320
GCC CTT AGG CCC CAG GGA CTG GCT AAG TCA GCG TCA CCC

Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Ala
TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AGC CTG CAG GCT
AGA CCC TGT CTA AAG TGA GAG TGG TAG TCG TCG GAC GTC CGA
```

FIG. 10 (CONT.)

```
                                        |────────CDR3────────
                                        ↓
Glu Asp Val Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Ser  93
GAA GAT GTG GCA GTT TAT TAC TGT CAG CAA TAT TAT AGT4400
CTT CTA CAC CGT CAA ATA ATG ACA GTC GTT ATA ATA TCA

          ↑
Tyr Pro Leu Thr Phe Gly Gly Thr Lys Val Val Ile  106
TAT CCT CTC ACT TTC GGC GGA ACC AAG GTG GTG AAA4440
ATA GGA GAG TGA AAG CCG CCT TGG TTC CAC CAC TAG

                                      Hind III
Lys A(rg)                                               107
AAA C GTAAGTACAC TTTTCTAAG CTT-3'                      4466
TTT G CATTCATGTG AAAAGATTC GAA 5'
```

FIG. 10 (CONT.)

FIGURE 10: Entire DNA sequence of the Hum4 V gene
Cla I-Hind III segment in pRL1001, Clone #2. A single
base difference occured at position 3461 and is marked
with an asterisk (*). The corresponding amino acid
sequences in the coding exons are shown. The site of
the Leu-Pro mutation in Clone #7 is boxed. An arrow ()
indicates the site of the single base deletion in Clone
#11. Oligonucleotides used as primers for the
sequencing reactions are underlined. In order as they
occur from the 5' end are: HUMLIN1(-), HUMLIN2(-),
HUMLCDR1(-) and Hind III Ck(-)(not shown).

FIG. 11
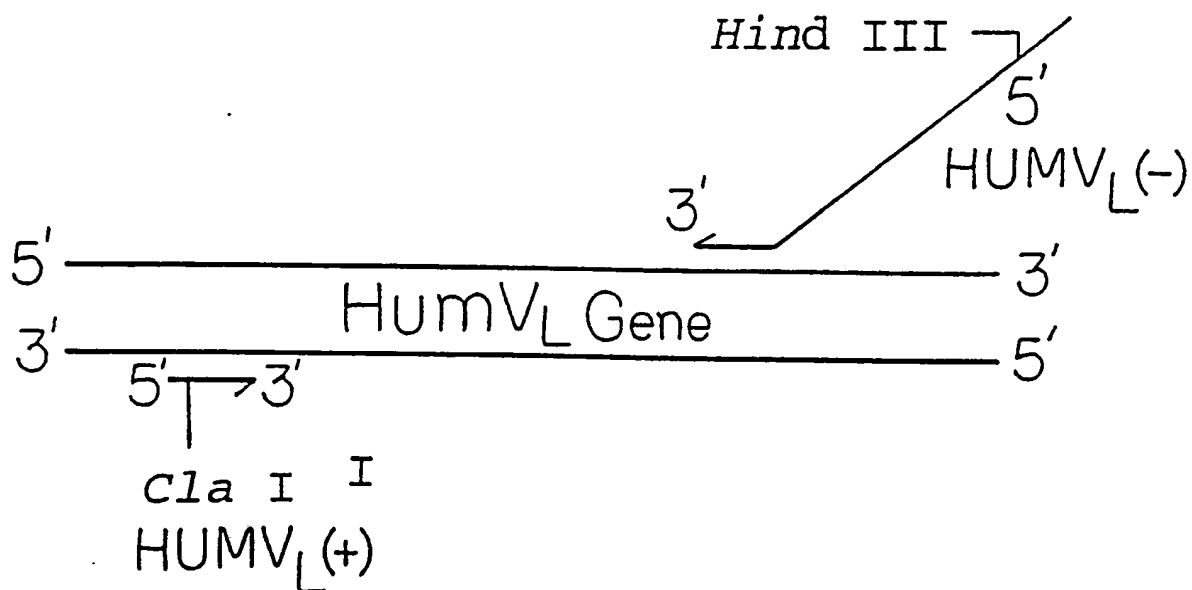
A schematic representation of the human germline Subgroup IV gene (HumV$_L$) as the target for the PCR. The 5'-end oligo (HUMVL(+)) and the 3'end oligo (HUMVL(-)) used to prime the elongation reactions for Taq polymerase are shown with half-arrows to indicate the direction of synthesis.

FIG. 12

1 2 3 4 5 6 7 8
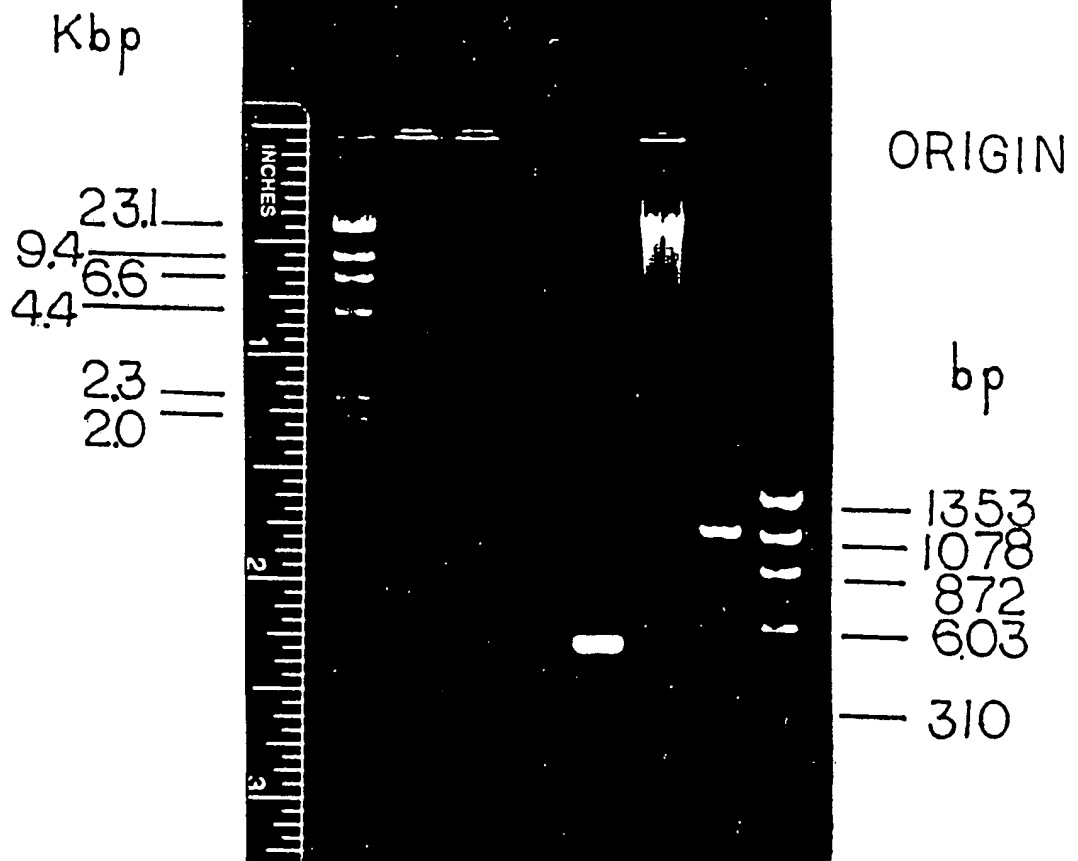
FIGURE 12: Agarose gel electrophoresis of Hum4V$_L$ PCR reactions. Lane 1: λ*Hind* III standard; lane 2: no Taq polymerase control; lane 3: no primers added; lane 4: no human DNA template; lane 5: Gene *Amp* kit positive control; lane 6: 3 μg human DNA with primers and Taq polymerase; lane 7: same as lane 6, but with 1ug human DNA and lane 8: ØX174-*Hae* III DNA standard. Ethidium bromide was added to the gel and buffer. Bands were visualized by long wavelength UV light.

Hind III, 555 FIG. 13

FIG. 14

```
                    Cla I   Eco RV   Spe I   Hind III   Bam HI
CH(+) 5'-GGATCATCGA TTGATATCAA CTAGTGAAGC TTATGGATCC
CH(-) 3'-CCTAGTAGCT AACTATAGTT GATCACTTCG AATACCTAGG
                  |_____ POLYLINKER _____|
        OLD Bam HI                              OLD Bam HI
        SITE FILED-                             SITE
        IN AND LOST                             FILLED-IN
                                                AND
                                                REGENERATED
```
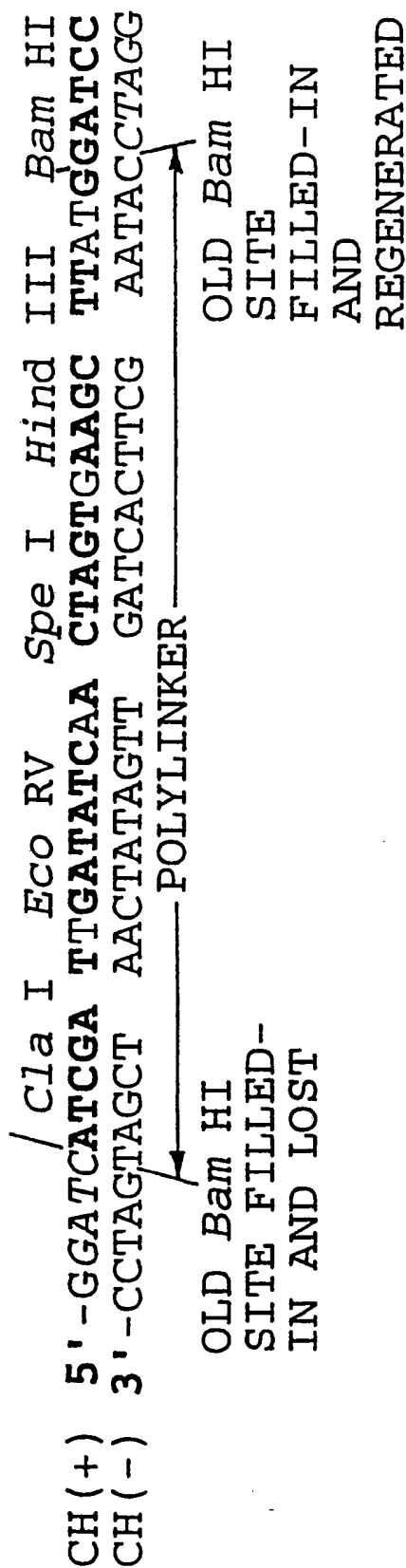
FIGURE 14: The polylinker inserted between filled-in Bam HI site of pSV2neo-101 to create pSV2neo-102. Note that the polylinker could be inserted in both orientations such that the Bam HI site on the left side could also be regenerated (and the one on the right side lost). The nucleotides used to fill-in the Bam HI site are shown in italics. The top synthetic oligo was called CH(+) while the complimentary strand was CH(-).

FIG. 15

(A portion of the DNA Sequence of pSV2neo)

————TOWARDS *Eco* RI SITE 5'-GAG<u>GAGGTTA</u>

<u>GGGTTTATGA</u> <u>GGAC</u>ACAGAG GAGCTTCCTG

GGGATCCAGA CATGATAAGA TACATTGATG
    Bam H1

AGTTTGGACA AACCACAACT AGA-3'

FIGURE 15: Oligonucleotide synthesized (21-mer, called NEO102SEQ) to sequence putative pSV2neo-102 clones is the underlined sequence shown above. The *Bam* HI site where the polylinker was inserted in pSV2neo-101 is boxed.

FIG. 16
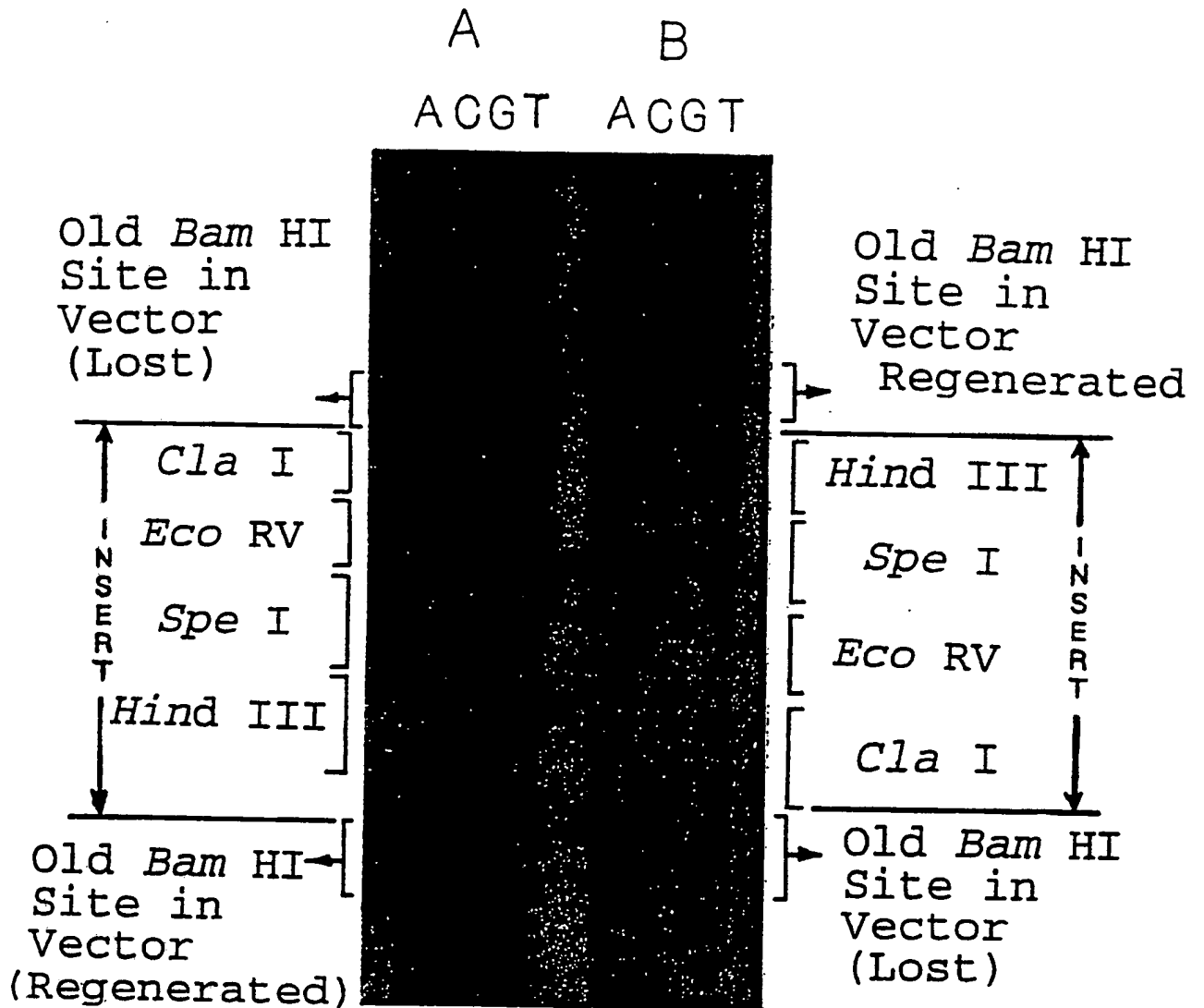
FIGURE 16: P-32 autoradiogram showing polylinker DNA sequence cloned in *Bam* HI site of pSV2neo-101. In both cases a single 30-base linker unit was incorporated, but in opposite orientations. Panel A-Sequence resulting in pSV2neo-120; Panel B-sequence resulting in pSV2neo-102. Reading the sequence (going up) is in the 5' to 3' direction of the (+) strand.

FIG. 17

LOST *Bam* HI
SITE IN
pSV2neo ─────┐
                     Cla I   *Eco* RV *Spe* I
5'-CTTCCTGGGG ATC**ATCGAT**T **GATATCAACT** 3394

                     FROM HUMAN C
                     Hind III-*Bam* HI INSERT
    Hind III
    AGTTGAAGCT **T**TTTTTTTTT CA<u>GTGCTATT</u> 3423

    <u>TAATTATTTC AATATCCTCT</u> CATCAAATGT 3453

    ATTTAAATAA CAAAAGCTCA ACCAAAAAGA 3483

    AAGAAATATG TAATTCTTTC AGAGTAAAAA 3513

    TCACACCCAT GACCTGGCCA CTGAGGGCTT 3543

    GATCAATTCA CTTTGAATTT GGCATTAAAT 3573

    ACCATTAAGG TATATTAACT GATTTTAAAA 3603
                                           TOWARDS
    TAAGATATAT TCGTGACC-3'   *Bam* HI   3621

FIGURE 17: DNA sequence from pRL1000,
reading the (+) strand from the primer
NEO102SEQ (Figure 15). Sequence data past
the *Hind* III site is from the human C$_K$
Hind III - *Bam* HI insert. The sequence
complementary to the underlined DNA
sequence, called *Hind* IIIC$_K$(-), was
synthesized as a primer for sequencing in
the upstream 3' direction.

FIG. 18

Nde I,559
Pvu I, 1999
Sca I,2109
Aat II,2551

Bam HI,9480

Neo^{R}

SV40
ori

Amp^{R}

20

pRL1001
11.82 Kbp

100

JVL

40

80

60

HUMAN C$_{k}$

EXON

Cla I,3379
Bam HI,3972
Kpn I, 4229
Hind III,
4460
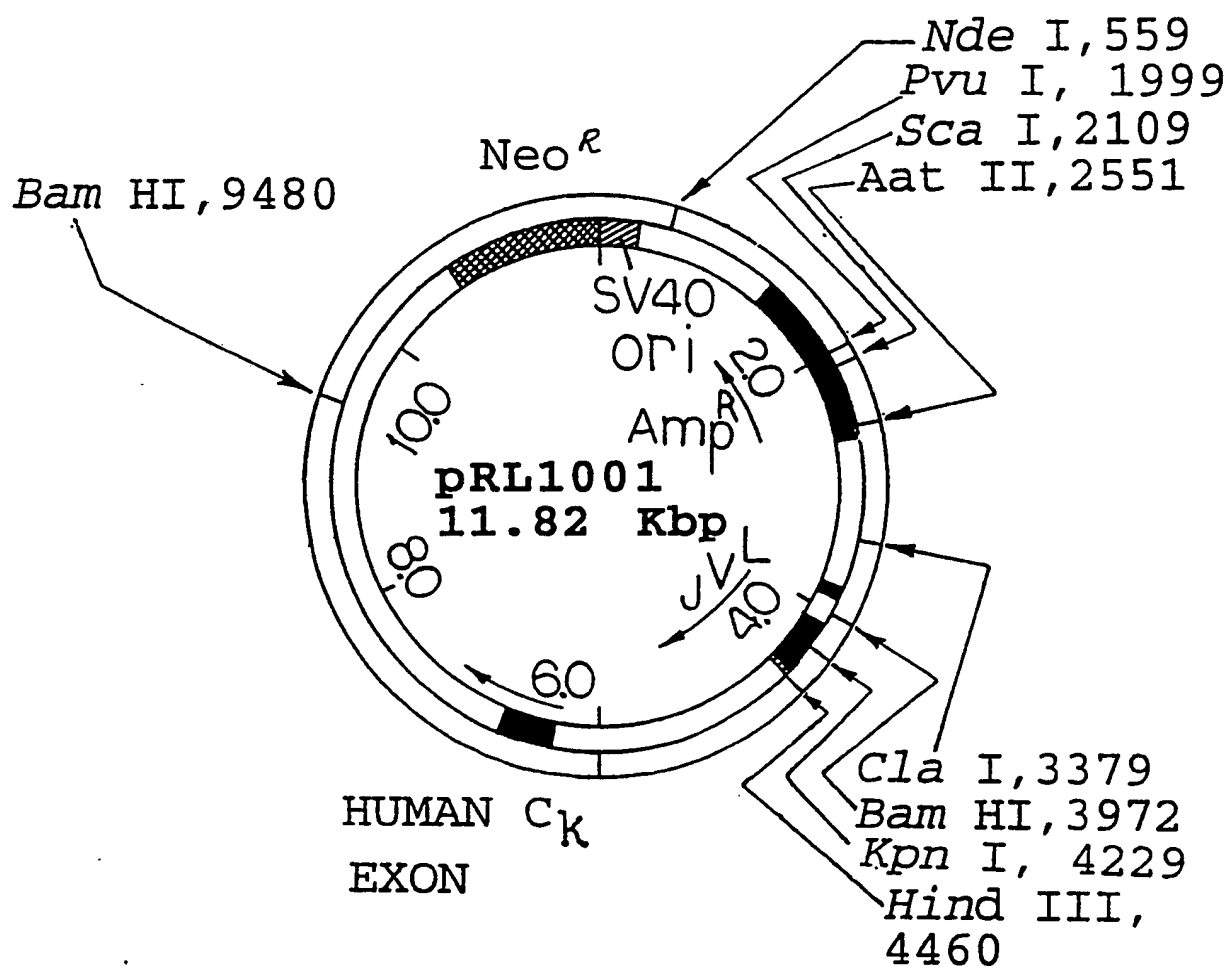
FIGURE 18: Partial restriction map of
the plasmid pRL1001. This is the
expression vector to introduce the
human anti-tumor L chain gene in Sp2/0
cells.

FIG. 19

DNA SEQUENCING – ρRL1001

CLONE	CLONE	CLONE	CLONE
2	7	9	11

A C G T   A C G T   A C G T   A C G T
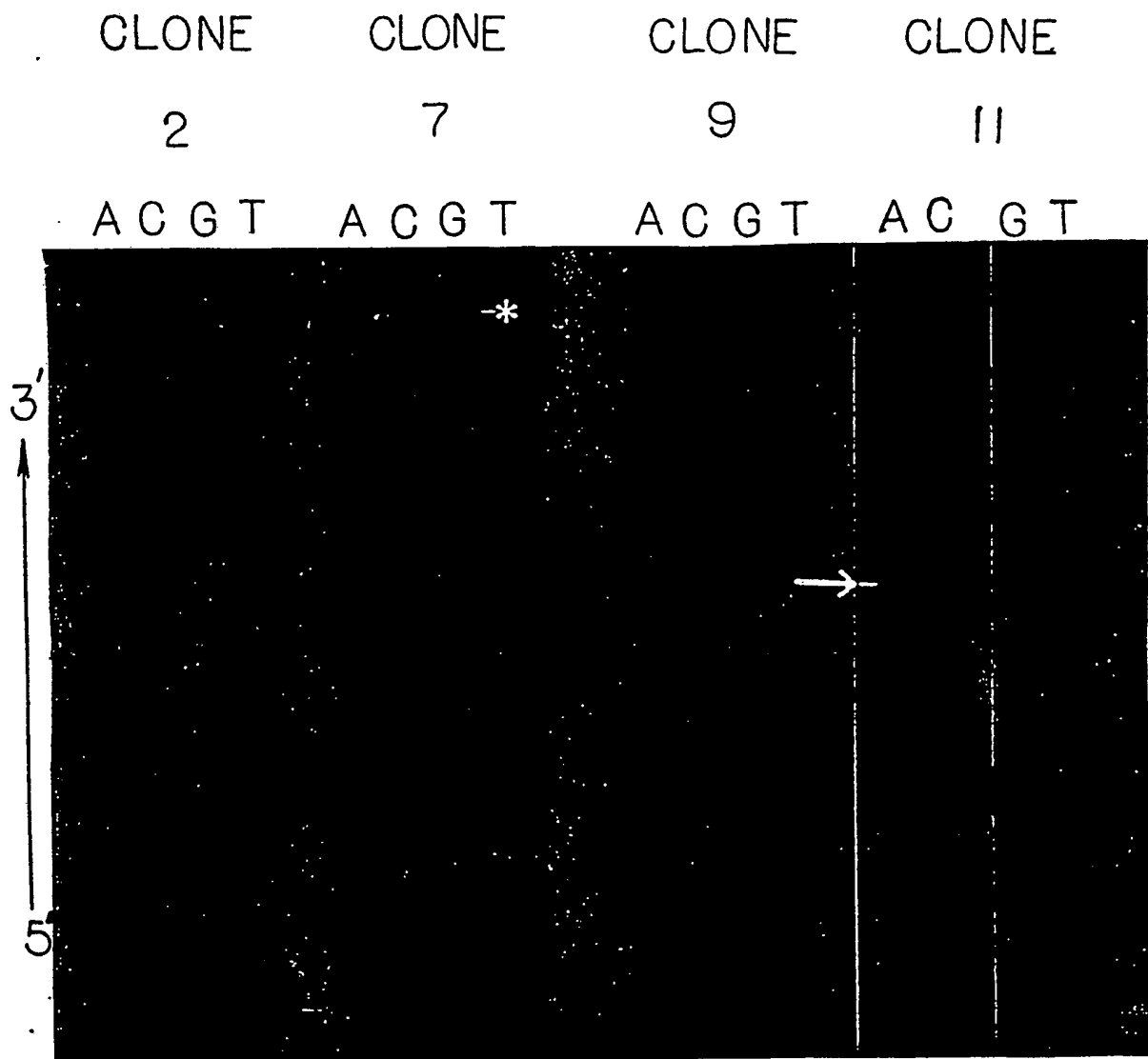
FIGURE 19: DNA sequence autoradiogram of pRL1001 clones. Reading the gel is in the 5' to 3' direction on the (-) strand, from the *Hin*d III C_K(-) primer. Clones 2 and 9 were equivalent to the expected sequence, clone 7 had a single base substitution (marked by *) and clone 11 had a single base deletion (marked by -)

Fig. 20

RECIPROCAL COMPETITION BETWEEN
CC49, Ch44, AND Ch44H

INHIBITION OF BINDING OF I-125 CC49 TO TAG-72

COMPETITOR

—□— CC49

—■— Ch44

—+— Ch44H

INHIBITION OF BINDING OF I-125 Ch44 TO TAG-72
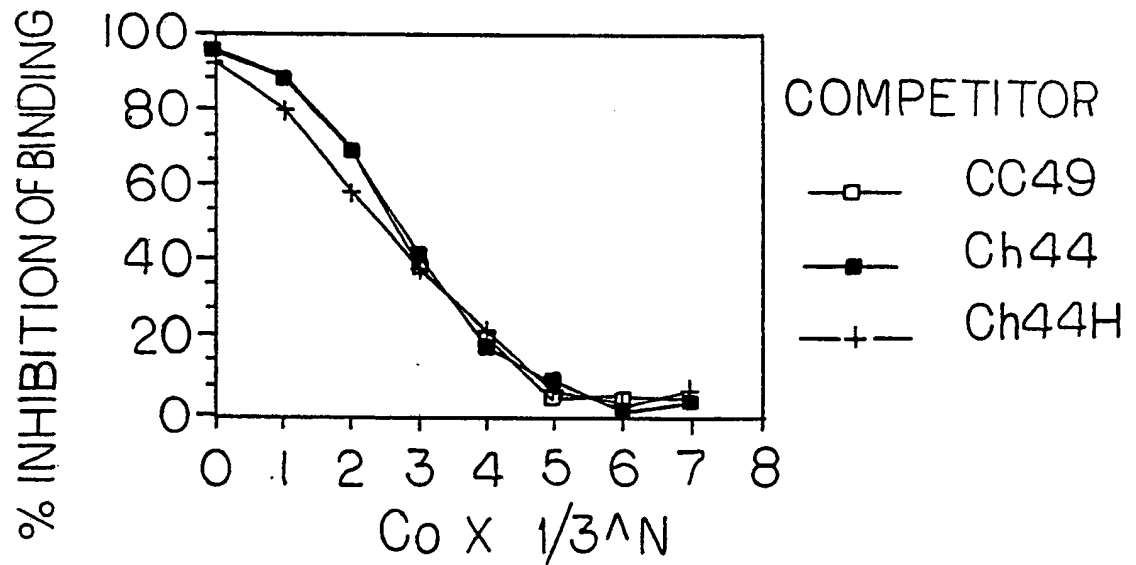
COMPETITOR
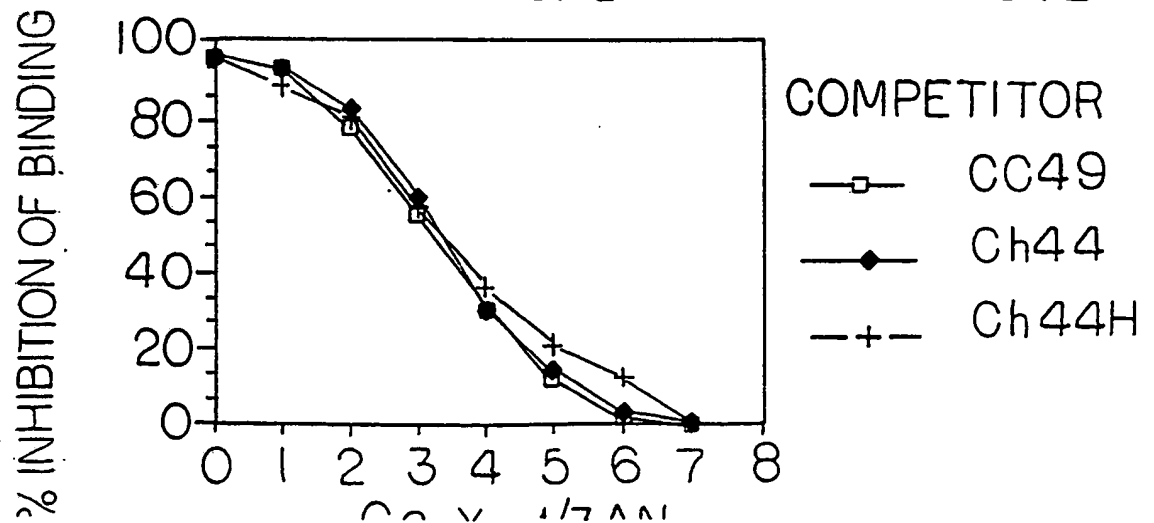
—□— CC49

—♦— Ch44

—+— Ch44H

Fig. 20 (CONT.)

RECIPROCAL COMPETITION BETWEEN CC49, Ch44, AND Ch44H

INHIBITION OF BINDING OF I-125 Ch44 TO TAG-72

COMPETITOR
—□— CC 49
—◆— Ch 44
—+— Ch 44H
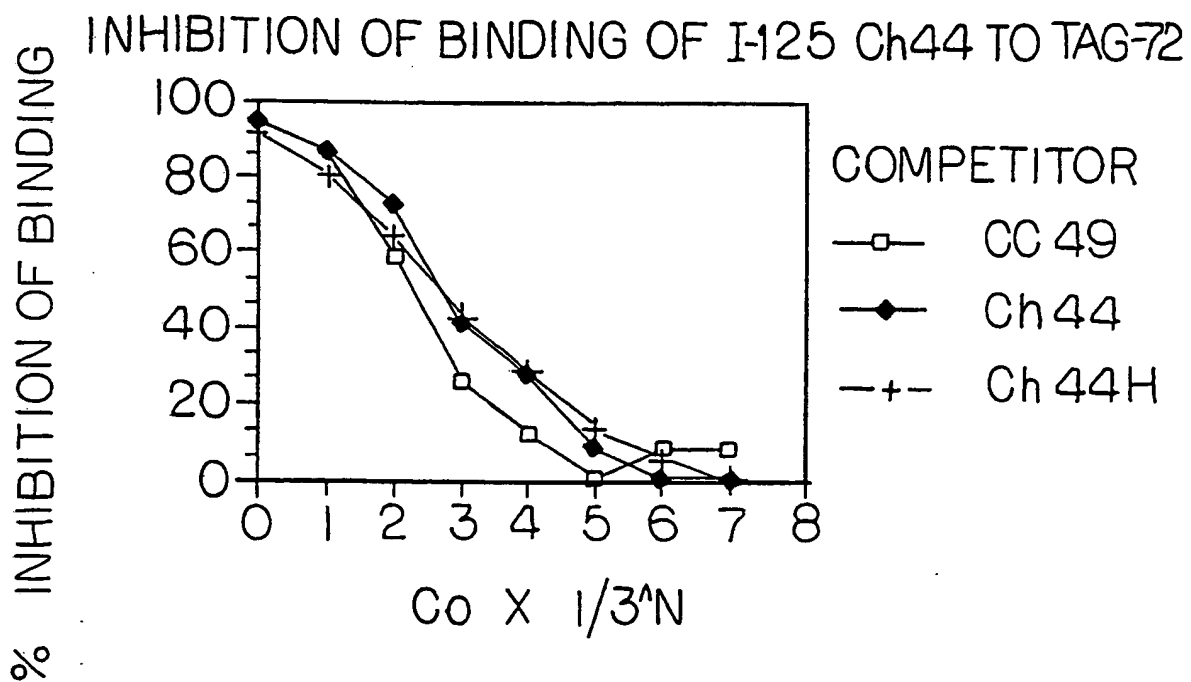
FIG. 21
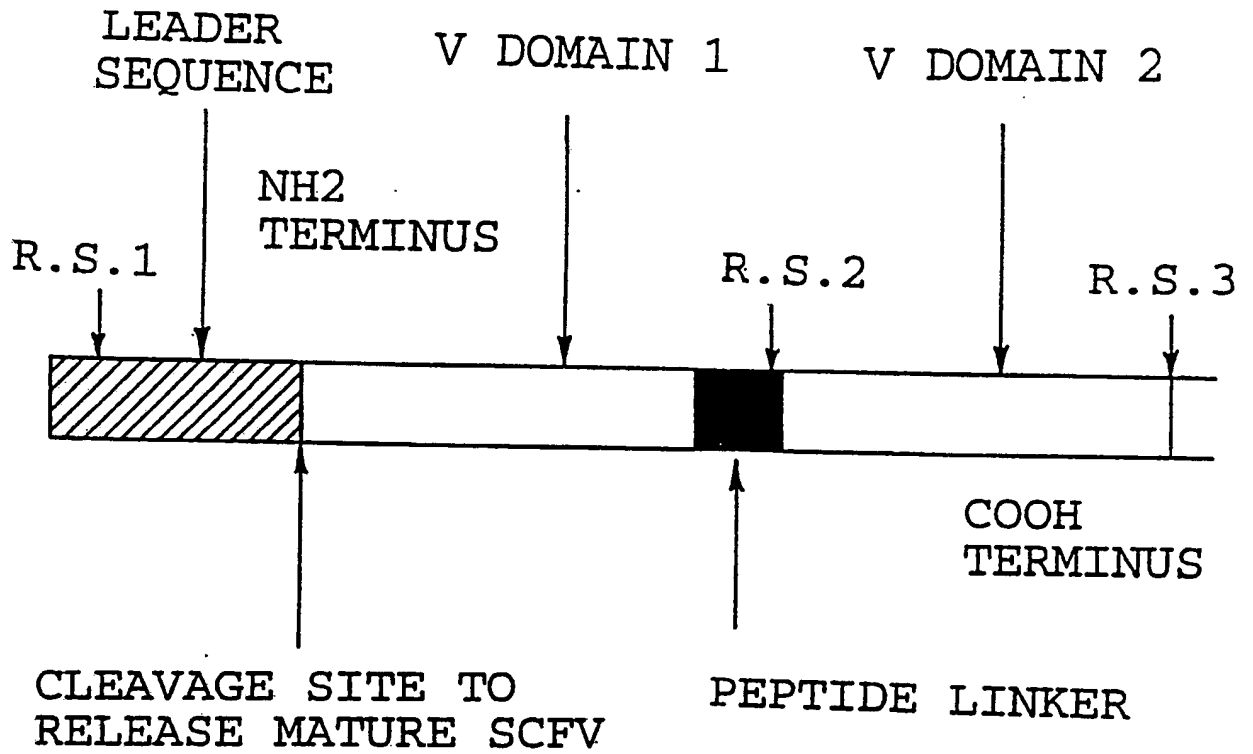
FIG. 22

```
                                            Met Leu
            AAAAACTAT AAGCTCCATG ATG CTT

Leu Gln Ala Phe Leu Phe Leu Leu Ala
TTG CAA GCT TTC CTT TTC CTT TTG GCT

Gly Phe Ala Ala Lys Ile Ser Ala Asp
GGT TTT GCA GCC AAA ATA TCT GCA GAC

Ile Val Met Thr Gln Ser Pro Asp Ser
ATC GTG ATG ACC CAG TCT CCA GAC TCC

Leu Ala Val Ser Leu Gly Glu Arg Ala
CTG GCT GTG TCT CTG GGC GAG AGG GCC
                                 CDR1L
Thr Ile Asn Cys Lys Ser Ser Gln Ser
ACC ATC AAC TGC AAG TCC AGC TGC AAG
```

```
Val Leu Tyr Ser Ser Asn Asn Lys Asn
GTT TTA TAC AGC TCC AAC AAT AAG AAC

Tyr Leu Ala Trp Tyr Gln Gln Lys Pro
TAC TTA GCT TGG TAC CAG CAG AAA CCA

Gly Gln Pro Pro Lys Leu Leu Ile Tyr
GGA CAG CCT CCT AAG CTG CTC ATT TAC
         CDR2L
Trp Ala Ser Thr Arg Glu Ser Gly Val
TGG GCA TCT ACC CGG GAA TCC GGG GTC

Pro Asp Arg Phe Ser Gly Ser Gly Ser
CCT GAC CGA TTC AGT GGC AGC GGG TCT
```

FIG. 22 (CONT.)

```
Gly Thr Asp Phe Thr Leu Thr Ile Ser
GGG ACA GAT TTC ACT CTC ACC ATC AGC

Ser Leu Gln Ala Glu Asp Val Ala Val
AGC CTG CAG GCT GAA GAT GTG GCA GTT
                 ┌────────────── CDR3L ──────────────
Tyr Tyr Cys │Gln Gln Tyr Tyr Ser Tyr
TAT TAC TGT │CAG CAA TAT TAT AGT TAT
    ────────────────→
Pro Leu Thr │Phe Gly Gly Gly Thr Lys
CCT CTC ACT │TTC GGC GGA GGG ACC AAG

Val │Lys Glu Ser Gly Ser Val Ser Ser
GTG │AAG GAG TCA GGT TCG GTC TCC TCA
       ──────── LINKER ────────────────
Glu Gln Leu Ala Gln Phe Arg Ser Leu
GAA CAA TTG GCC CAA TTT CGT TCC TTA
──────→
Asp │Val Gln Leu Gln Gln Ser Asp Ala
GAC │GTC CAG TTG CAG CAG TCT GAC GCT

Glu Leu Val Lys Pro Gly Ala Ser Val
GAG TTG GTG AAA CCT GGG GCT TCA GTG

Lys Ile Ser Cys Lys Ala Ser Gly Tyr
AAG ATT TCC TGC AAG GCT TCT GGC TAC
                 ┌────────────── CDR1H ──────────────→
Thr Phe Thr │Asp His Ala Ile His │Trp
ACC TTC ACT │GAC CAT GCA ATT CAC │TGG

Val Lys Gln Asn Pro Glu Gln Gly Leu
GTG AAA CAG AAC CCT GAA CAG GGC CTG
```

FIG. 22 (CONT.)

```
Glu Trp Ile Gly │Tyr Phe Ser Pro Gly
GAA TGG ATT GGA │TAT TTT TCT CCC GGA
─────────────────────── CDR2H ───────────
Asn Asp Asp Phe Lys Tyr Asn Glu Arg
AAT GAT GAT TTT AAA TAC AAT GAG AGG

Phe Lys Gly │Lys Ala Thr Leu Thr Ala
TTC AAG GGC │AAG GCC ACA CTG ACT GCA

Asp Lys Ser Ser Ser Thr Ala Tyr Val
GAC AAA TCC TCC AGC ACT GCC TAC GTG

Gln Leu Asn Ser Leu Thr Ser Glu Asp
CAG CTC AAC AGC CTG ACA TCT GAG GAT

Ser Ala Val Tyr Phe Cys Thr Arg │Ser
TCT GCA GTG TAT TTC TGT ACA AGA │TCC
──────── CDR3H ────────────────
Leu Asn Met Ala Tyr │Trp Gly Gln Gly
CTG AAT ATG GCC TAC │TGG GGT CAA GGA

Thr Ser Val Thr Val Ser
ACC TCA GTC ACC GTC TCC TAG TGA
```

AGCTTGGAAC ACCACACAAA CCATATCCAA A

Fig. 23

Fig. 24

Fig. 24 (CONT.)

Hind III

Nde I

AMP^R

LEU2

Nco I

**pCGS515/SCFV1
11.5Kbp**

URA3

Sal I

SCFV1

Cla I

Hind III

Aat II

Hind III

PCR WITH OLIGOS
penP3 + penP6(-)

Aat II

Nco I   *Bcl I*

Hind III   *Aat II*

Nco I   *Bcl I*

V_L V_H

V_L E V_H

PCR/SOE with
FRAGMENT B
USING OLIGOS
penP1 + penP6(-)

LIGATE WITH
FRAGMENTS
A + C

Bcl I

Nco I

Eco RI

Cam^R

Hind III

SCFV

V_L

Nco I

Aat II

V_H

Bcl I

**pSCFV31
6.0Kbp**

Bam HI

Sal I

FIG. 25

FIG. 26

CTCATGTTTG ACAGCTTATC ATCGAT**GAAT**

***Eco* RI**

TCCATCACTT CCCTCCGTTC ATTTGTCCCC

GGTGGAAACG AGGTCATCAT TTCCTTCCGA

AAAAACGGTT ·GCATTTAAAT CTTACATATG

TAATACTTTC AAAGACTACA TTTGTAAGAT

TTGATGTTTG AGTCGGCTGA AAGATCGTAC

GTACCAATTA TTGTTTCGTG ATTGTTCAAG

CCATAACACT GTAGGGATAG TGGAAAGAGT

GCTTCATCTG GTTACGATCA ATCAAATATT

			***pelB* Signal**		
			Met	Lys	Tyr	Leu
CAAACGGAGG	GAGACGATTT	TG| ATG	AAA	TAC	CTA

Sequence

Leu	Pro	Thr	Ala	Ala	Ala	Gly	Leu	Leu	Leu
TTG	CCT	ACG	GCA	GCC	GCT	GGA	TTG	TTA	TTA

					***Nco* I** →	←	**H4V**_L_
Leu	Ala	Ala	Gln	Pro	Ala	Met	Ala| Asp	Ile
CTC	GCT	GCC	CAA	CCA	**GCC**	**ATG**	**GCC**| GAC	ATC

Val	Met	Thr	Gln	Ser	Pro	Asp	Ser	Leu	Ala
GTG	ATG	ACC	CAG	TCT	CCA	GAC	TCC	CTG	GCT

Val	Ser	Leu	Gly	Glu	Arg	Ala	Thr	Ile	Asn
GTG	TCT	CTG	GGC	GAG	AGG	GCC	ACC	ATC	AAC

Cys	Lys	Ser	Ser	Gln	Ser	Val	Leu	Tyr	Ser
TGC	AAG	TCC	AGC	CAG	AGT	GTT	TTA	TAC	AGC

FIG. 26 (CONT.)

```
Ser Asn Asn Lys Asn Tyr Leu Ala Trp Tyr
TCC AAC AAT AAG AAC TAC TTA GCT TGG TAC

Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu
CAG CAG AAA CCA GGA CAG CCT CCT AAG CTG

Leu Ile Tyr Trp Ala Ser Thr Arg Glu Ser
CTC ATT TAC TGG GCA TCT ACC CGG GAA TCC

Gly Val Pro Asp Arg Phe Ser Gly Ser Gly
GGG GTC CCT GAC CGA TTC AGT GGC AGC GGG

Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC

Ser Leu Gln Ala Glu Asp Val Ala Val Tyr
AGC CTG CAG GCT GAA GAT GTG GCA GTT TAT

Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu
TAC TGT CAG CAA TAT TAT AGT TAT CCT CTC
```

H4V_L

```
Thr Phe Gly Gly Gly Thr Lys Val Val Ile
ACT TTC GGC GGA GGG ACC AAG GTG GTG ATC
```

***Hind* III LINKER**

```
Lys│Leu  Ser Ala Asp Asp Ala Lys Lys Asp
AAG│CTT AGT GCG GAC GAT GCG AAA AAG GAT

Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp
GCT GCG AAG AAG GAT GAC GCT AAG AAA GAC
```

LINKER   *Xho* I CC49 V_H

```
Asp Ala Lys Lys Asp Leu│Glu Val Gln Leu
GAT GCT AAA AAG GAC CTC│GAG GTT CAG TTG
```

FIG. 26 (CONT.)

```
Gln Gln Ser Ala Glu Leu Val Lys Pro Gly
CAG CAG TCT GCT GAG TTG GTG AAA CCT GGG

Ala Ser Val Lys Ile Ser Cys Lys Ala Ser
GCT TCA GTG AAG ATT TCC TGC AAG GCT TCT

Gly Tyr Thr Phe Thr Asp His Ala Ile His
GGC TAC ACC TTC ACT GAC CAT GCA ATT CAC

Trp Val Lys Gln Asn Pro Glu Gln Gly Leu
TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG

Glu Trp Ile Gly Tyr Phe Ser Pro Gly Asn
GAA TGG ATT GGA TAT TTT TCT CCC GGA AAT

Asp Asp Phe Lys Tyr Asn Glu Arg Phe Lys
GAT GAT TTT AAA TAC AAT GAG AGG TTC AAG

Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser
GGC AAG GCC ACA CTG ACT GCA GAC AAA TCC

Ser Ser Thr Ala Tyr Val Gln Leu Asn Ser
TCC AGC ACT GCC TAC GTG CAG CTC AAC AGC

Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe
CTG ACA TCT GAG GAT TCT GCA GTG TAT TTC

Cys Thr Arg Ser Leu Asn Met Ala Tyr Trp
TGT ACA AGA TCC CTG AAT ATG GCC TAC TGG
                            CC49 VH ──────────→
Gly Gln Gly Thr Ser Val Thr Val Ser Ser
GGT CAA GGA ACC TCA GTC ACC GTC TCC TCA
```

FIG. 26 (CONT.)

Nhe I
TAA AAA**GCTAGC**G ATGAATCCGT CAAAACATCA

Bcl I
.TCTTACATAA AGTCACTTGG **TGATCA**AGCT

CATATCATTG TCCGGCAATG GTGTGGGCTT

TTTTTGTTTT CTATCTTTAA AGATCATGTG

AAGGAAAAAA CGGGAAAATC GGTCTGCGGG

AAAGGACCGG GTTTTTGTCG AAATCATAGG

Bam HI
CGAATGGGTT GGATTGTGAC AAAATTC**GGA TCC**

FIG. 27

CONSTRUCTION OF PLASMID pSCFV UHH. GENERATION OF A
COMBINATORIAL LIBRARY OF V$_H$ GENES WITH HUM4 V$_L$

pSCFV UNIH

1. DIGEST WITH *Hind* III +*Xho* I
   PURIFY AWAY L

2. LIGATE WITH UNIHOPE
   Hind III/*Xho* I L

1. DIGEST WITH
   Xho I+*Nhe* I.
   PURIFY AWAY
   CC49 V$_H$

2. LIGATE WITH
   HUMAN V$_H$
   Xho I/*Nhe* I
   PCR DNA
   INSERTS

COMBINATORIAL LIBRARY OF HUMAN
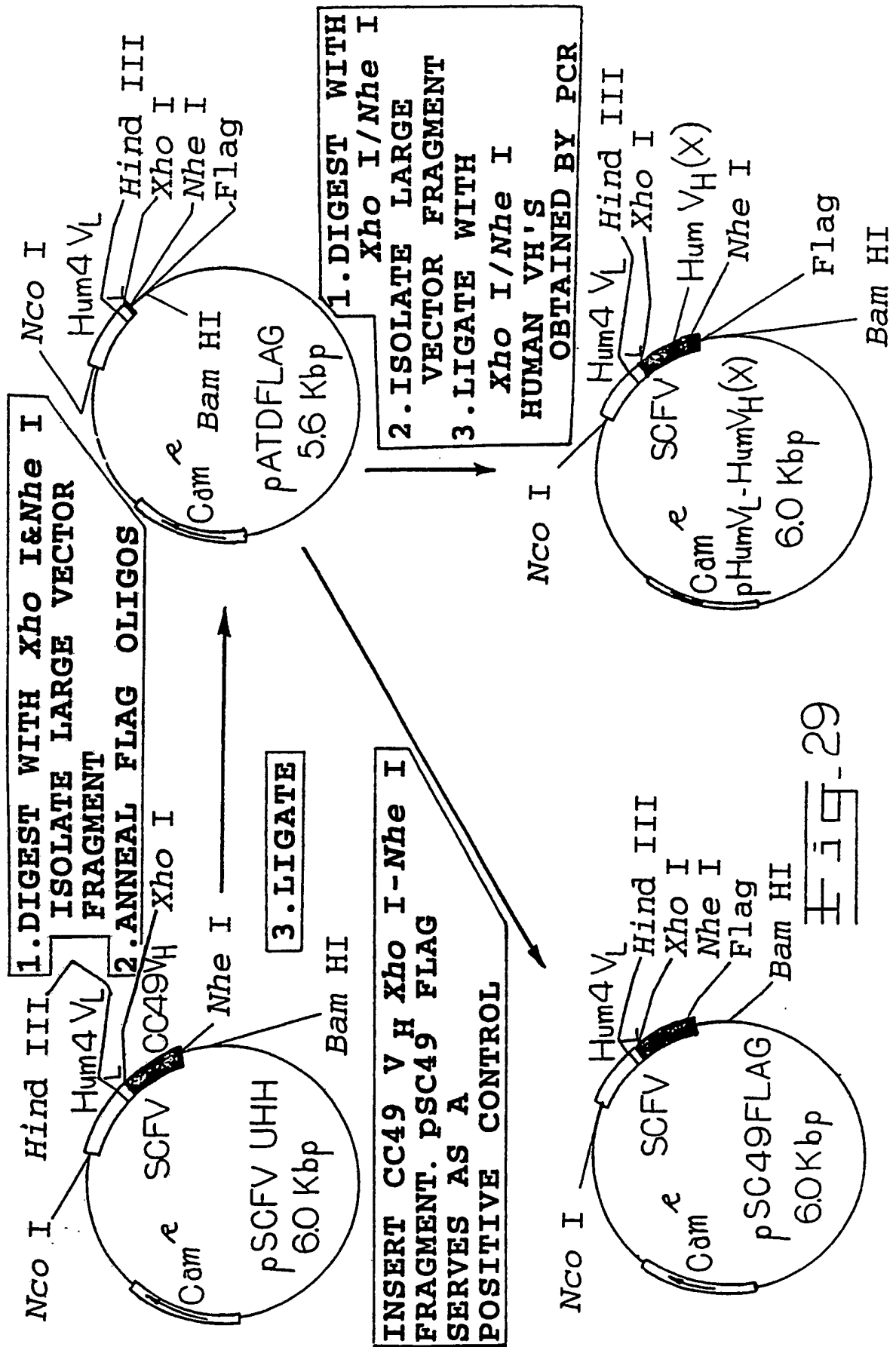V$_H$ GENES WITH HUMAN SUBGROUP IV V$_L$
EXPRESSED AS SCFV PRODUCTS

FIG. 28

CTCATGTTTG ACAGCTTATC ATCGAT**GAAT**

TCCATCACTT CCCTCCGTTC ATTTGTCCCC

GGTGGAAACG AGGTCATCAT TTCCTTCCGA

AAAAACGGTT GCATTTAAAT CTTACATATG

TAATACTTTC AAAGACTACA TTTGTAAGAT

TTGATGTTTG AGTCGGCTGA AAGATCGTAC

GTACCAATTA TTGTTTCGTG ATTGTTCAAG

CCATAACACT GTAGGGATAG TGGAAAGAGT

GCTTCATCTG GTTACGATCA ATCAAATATT

```
                                pelB Signal
                           Met Lys Tyr Leu
CAAACGGAGG GAGACGATTT TG|ATG AAA TAC CTA
Sequence
Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu
TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA
                        Nco I ─→|←── H4V_L
Leu Ala Ala Gln Pro Ala  Met Ala|Asp Ile
CTC GCT GCC CAA CCA GCC ATG GCC|GAC ATC

Val Met Thr Gln Ser Pro Asp Ser Leu Ala
GTG ATG ACC CAG TCT CCA GAC TCC CTG GCT

Val Ser Leu Gly Glu Arg Ala Thr Ile Asn
GTG TCT CTG GGC GAG AGG GCC ACC ATC AAC

Cys Lys Ser Ser Gln Ser Val Leu Tyr Ser
TGC AAG TCC AGC CAG AGT GTT TTA TAC AGC
```

FIG. 28 (CONT.)

```
Ser Asn Asn Lys Asn Tyr Leu Ala Trp Tyr
TCC AAC AAT AAG AAC TAC TTA GCT TGG TAC

Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu
CAG CAG AAA CCA GGA CAG CCT CCT AAG CTG

Leu Ile Tyr Trp Ala Ser Thr Arg Glu Ser
CTC ATT TAC TGG GCA TCT ACC CGG GAA TCC

Gly Val Pro Asp Arg Phe Ser Gly Ser Gly
GGG GTC CCT GAC CGA TTC AGT GGC AGC GGG

Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC

Ser Leu Gln Ala Glu Asp Val Ala Val Tyr
AGC CTG CAG GCT GAA GAT GTG GCA GTT TAT

Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu
TAC TGT CAG CAA TAT TAT AGT TAT CCT CTC
                                        H4V_L
Thr Phe Gly Gly Gly Thr Lys Val Val Ile
ACT TTC GGC GGA GGG ACC AAG GTG GTG ATC
Hind III   LINKER
Lys|Leu   Ser Ala Asp Asp Ala Lys Lys Asp
AAG|CTT AGT GCG GAC GAT GCG AAA AAG GAT

Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp
GCT GCG AAG AAG GAT GAC GCT AAG AAA GAC
              LINKER   Xho I
Asp Ala Lys Lys Asp Leu|Glu
GAT GCT AAA AAG GAC CTC|GAG
```

FIG. 28 (CONT.)

```
         Nhe I  | Flag Peptide
      Ala   Ser| Asp Tyr Lys Asp
ACAATGTC GCT AGC|GAC TAC AAG GAC
```

```
Asp Asp Asp Lys|
GAT GAT GAC AAA|TAA  AAACCTAGC
```

```
GATGAATCCG TCAAAACATC ATCTTACATA
              Bcl I
AAGTCACTT GGTGATCAAG CTCATATCAT

TGTCCGGCA ATGGTGTGGG CTTTTTTTGT

TTTCATCTT TAAAGATCAT GTGAAGGAAA

AAACGGGAA AATCGGTCTG CGGGAAAGGA

CCGGGTTTT TGTCGAAATC ATAGGCGAAT
                        Bam HI
GGGTTGGAT TGTGACAAAA TTCGGATCC
```

Fig-29

FIG. 30

Eco **RI**

CTCATGTTTG ACAGCTTATC ATCGAT**GAAT**

TCCATCACTT CCCTCCGTTC ATTTGTCCCC

GGTGGAAACG AGGTCATCAT TTCCTTCCGA

AAAAACGGTT GCATTTAAAT CTTACATATG

TAATACTTTC AAAGACTACA TTTGTAAGAT

TTGATGTTTG AGTCGGCTGA AAGATCGTAC

GTACCAATTA TTGTTTCGTG ATTGTTCAAG

CCATAACACT GTAGGGATAG TGGAAAGAGT

GCTTCATCTG GTTACGATCA ATCAAATATT

                                        pelB **Signal**
                                  Met Lys Tyr Leu
CAAACGGAGG GAGACGATTT TG│ATG AAA TAC CTA
Sequence
Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu
TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA
                          Nco **I** ──►│◄── **H4V**$_L$
Leu Ala Ala Gln Pro Ala Met Ala│Asp Ile
CTC GCT GCC CAA CCA **GCC ATG GCC**│GAC ATC

Val Met Thr Gln Ser Pro Asp Ser Leu Ala
GTG ATG ACC CAG TCT CCA GAC TCC CTG GCT

Val Ser Leu Gly Glu Arg Ala Thr Ile Asn
GTG TCT CTG GGC GAG AGG GCC ACC ATC AAC

Cys Lys Ser Ser Gln Ser Val Leu Tyr Ser
TGC AAG TCC AGC CAG AGT GTT TTA TAC AGC

FIG. 30 (CONT.)

```
Ser Asn Asn Lys Asn Tyr Leu Ala Trp Tyr
TCC AAC AAT AAG AAC TAC TTA GCT TGG TAC

Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu
CAG CAG AAA CCA GGA CAG CCT CCT AAG CTG

Leu Ile Tyr Trp Ala Ser Thr Arg Glu Ser
CTC ATT TAC TGG GCA TCT ACC CGG GAA TCC

Gly Val Pro Asp Arg Phe Ser Gly Ser Gly
GGG GTC CCT GAC CGA TTC AGT GGC AGC GGG

Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC

Ser Leu Gln Ala Glu Asp Val Ala Val Tyr
AGC CTG CAG GCT GAA GAT GTG GCA GTT TAT

Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu
TAC TGT CAG CAA TAT TAT AGT TAT CCT CTC
                                        H4V_L
Thr Phe Gly Gly Gly Thr Lys Val Val Ile
ACT TTC GGC GGA GGG ACC AAG GTG GTG ATC
```

Hind III LINKER
```
Lys|Leu  Ser Ala Asp Asp Ala Lys Lys Asp
AAG|CTT AGT GCG GAC GAT GCG AAA AAG GAT

Ala Ala Lys Lys Asp Asp Ala Lys Lys Asp
GCT GCG AAG AAG GAT GAC GCT AAG AAA GAC
```
              LINKER   _Xho_ I   CC49 V_H
```
Asp Ala Lys Lys Asp Leu|Glu Val Gln Leu
GAT GCT AAA AAG GAC CTC|GAG GTT CAG TTG
```

FIG. 30 (CONT.)

```
Gln Gln Ser Ala Glu Leu Val Lys Pro Gly
CAG CAG TCT GCT GAG TTG GTG AAA CCT GGG

Ala Ser Val Lys Ile Ser Cys Lys Ala Ser
GCT TCA GTG AAG ATT TCC TGC AAG GCT TCT

Gly Tyr Thr Phe Thr Asp His Ala Ile His
GGC TAC ACC TTC ACT GAC CAT GCA ATT CAC

Trp Val Lys Gln Asn Pro Glu Gln Gly Leu
TGG GTG AAA CAG AAC CCT GAA CAG GGC CTG

Glu Trp Ile Gly Tyr Phe Ser Pro Gly Asn
GAA TGG ATT GGA TAT TTT TCT CCC GGA AAT

Asp Asp Phe Lys Tyr Asn Glu Arg Phe Lys
GAT GAT TTT AAA TAC AAT GAG AGG TTC AAG

Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser
GGC AAG GCC ACA CTG ACT GCA GAC AAA TCC

Ser Ser Thr Ala Tyr Val Gln Leu Asn Ser
TCC AGC ACT GCC TAC GTG CAG CTC AAC AGC

Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe
CTG ACA TCT GAG GAT TCT GCA GTG TAT TTC

Cys Thr Arg Ser Leu Asn Met Ala Tyr Trp
TGT ACA AGA TCC CTG AAT ATG GCC TAC TGG
```

CC49 V$_H$ ⟶

```
Gly Gln Gly Thr Ser Val Thr Val Ser Ser
GGT CAA GGA ACC TCA GTC ACC GTC TCC TCA
```

FIG. 30 (CONT.)

```
    Nhe I     | Flag Peptide
  Ala   Ser | Asp Tyr Lys Asp
  GCT   AGC | GAC TAC AAG GAC

Asp Asp Asp Lys|
GAT GAT GAC AAA| TAA   AAACCTAGC

GATGAATCCG TCAAAACATC ATCTTACATA
         Bcl I
AAGTCACTT GGTGATCAAG CTCATATCAT

TGTCCGGCA ATGGTGTGG  CTTTTTTTGT

TTTCATCTT TAAAGATCAT GTGAAGGAAA

AAACGGGAA AATCGGTCTG CGGGAAAGGA

CCGGGTTTT TGTCGAAATC ATAGGCGAAT
                          Bam HI
GGGTTGGAT TGTGACAAAA TTCGGATCC
```

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member		
AU 89/43540	AU 44299/89	BR 8907126	CA 2000913
	DK 1499/90	EP 365997	EP 397821
	FI 903056	HU 896255	IL 92037
	JP 3502889	NO 902696	WO 9004410
	ZA 8907858		
AU 89/44299	AU 43540/89	BR 8907126	CA 2000913
	DK 1499/90	EP 397821	EP 365997
	FI 903056	HU 896255	HU 56878
	IL 92037	JP 3502889	NO 902696
	WO 9004410	ZA 8907858	

International Application No: PCT/ AU91 /00583

MICROORGANISMS

Optional Sheet In connection with the microorganism referred to on page____46____ line____3____ of the description ¹

A. IDENTIFICATION OF DEPOSIT ¹

Further deposits are identified on an additional sheet ☐ ²

Name of depositary Institution ⁴

American Type Culture Collection

Address of depositary Institution (including postal code and country) ⁴

12301 Parklawn Drive,
Rockville, Maryland 20852 USA

Date of deposit ⁴	Accession Number ⁴
April 18, 1990	ATCC HB 10426

B. ADDITIONAL INDICATIONS ¹ (leave blank if not applicable). This Information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE ¹ (if the indications are not for all designated States)

The cell lines will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a United States Patent is issued citing the cell lines.

D. SEPARATE FURNISHING OF INDICATIONS ¹ (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later ¹ (Specify the general nature of the indications e.g., "Accession Number of Deposit").
In accordance with Regulation 3.25 of the Patents Regulations (Australia Statutory Rules 1991 No 71), samples of materials deposited in accordance with the Budapest Treaty in relation to an Australian Patent Request are only to be provided before: the patent is granted on the application; or the application has lapsed or been withdrawn or refused; to a person who is: a skilled addressee without an interest in the invention; and nominated by a person who makes a request for the furnishing of those samples.

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

_____
(Authorized Officer)

☒ The date of receipt (from the applicant) by the International Bureau ¹⁰

·-· 27 JAN 1992

_____
(Authorized Officer)

Form PCT/RO/134 (January 1981)

International Application No: PCT/ AU91 / 00583

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 46 line 3 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet ☐

Name of depositary institution

American Type Culture Collection

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852 USA

Date of deposit	Accession Number
April 18, 1990	ATCC HB 10427

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

The cell lines will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a United States Patent is issued citing the cell lines.

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g. "Accession Number of Deposit")

In accordance with Regulation 3.25 of the Patents Regulations (Australia Statutory Rules 1991 No 71), samples of materials deposited in accordance with the Budapest Treaty in relation to an Australian Patent Request are only to be provided before: the patent is granted on the application; or the application has lapsed, or been withdrawn or refused; to a person who is: a skilled addressee without an interest in the invention; and nominated by a person who makes a request for the furnishing of those samples.

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

_____
(Authorized Officer)

☒ The date of receipt (from the applicant) by the International Bureau

27 JAN 1992

_____
(Authorized Officer)

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent classification (IPC) or to both National Classification and IPC
Int. Cl.[5] C12N 15/13, C12N 5/10, A61K 39/395, 47/48, 49/02, 43/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	WPAT, Chemical Abstracts, Derwent Databases: Keywords: TAG 72 or (tumor or tumour) and associated and glyco:

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [8]

AU Search: C12N 15/13, C12P 21/08 Biotechnology Abstracts Derwent Database: Keywords: TAG 72 or (tumor or tumour) and associated and glyco:

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category[*]	Citation of Document, [11] with indication, where appropriate of the relevant passages [12]	Relevant to Claim No [13]
X	AU,A, 43540/89 (THE DOW CHEMICAL COMPANY) 26 April 1990 (26.04.90). See claims 1, 2, 5-9, 11-14, 16, 27, 29, 30, 32-35, 37-39	1-33
X	AU,A, 44299/89 (THE DOW CHEMICAL COMPANY) 14 May 1990 (14.05.90). See claims 1, 7-11, 13-18, 29, 31-38, 48-50	1-3, 5-33
Y	Whittle, N et al: 'Expression in COS cells of a mouse-human chimaeric B72.3 antibody'. Protein Engineering, volume 1, no. 6, pages 499-505, 1987	1, 6-9
Y	Brady, R L et al.: "Crystallization and Preliminary X-ray Diffraction Study of a Chimaeric Fab' Fragment of Antibody (continued)	1, 6-9

*	Special categories of cited documents : [10]	"T" Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	Document defining the general state of the art which is not considered to be of particular relevance	
"E"	earlier document but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	
"P"	document published prior t the international filing date but later than the priority date claimed	"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30 July 1992 (30.07.92)	21 Aug 1992 (21.08.92)

International Searching Authority	Signature of Authorized Officer
AUSTRALIAN PATENT OFFICE	K AYERS

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Binding Tumour Cells". J Mol Biol, 219, pages 603-604, 1991

V. ☐ **OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE** [1]

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4a

---

VI. ☐ **OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING** [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.